REMARKS

I. Status Summary

Claims 1-60 are pending in the present application and claims 1-4 are currently examined. Claims 5-60 are withdrawn due to restriction requirement. Claims 1-4 are herein amended.

The Examiner has made an objection to claim 4. In addition, claims 2-4 are rejected under 35 USC § 112, second paragraph, as allegedly being indefinite. Claims 1-4 are rejected under 35 USC § 112, first paragraph, as allegedly failing to enable a person of skill in the art to use the invention and allegedly failing to adequately describe that the inventors had possession of the invention at the time of filing.

II. Claim Amendments

Claim 1 has been amended as follows: "An isolated and purified biologically active heparan sulfate 3-*O*-sulfotransferase 5 polypeptide having greater than 95% sequence identity to SEQ ID NO 2". Support for this amendment can be found throughout the claims and specification as filed and, in particular, at page 21, lines 15-20 of the specification. Accordingly, no impermissible new matter has been added by this claim amendment.

Claim 2 is amended at part (b) to recite 95% rather than 90% sequence identity. Support for this amendment can be found throughout the claims and specification as filed and, in particular, at page 21, lines 10-15 of the specification. Claim 2 is further amended by deletion of parts (d) and (e) and has been converted to a Markush-type format. Accordingly, no impermissible new matter has been added by this claim amendment.

Claim 3 is amended to recite "the polypeptide is a human heparan sulfate 3-*O*-sulfotransferase 5 polypeptide" rather than the polypeptide "comprises" a human heparan sulfate 3-*O*-sulfotransferase 5 polypeptide. Accordingly, no impermissible new matter has been added by this claim amendment.

Claim 4 is amended such that the use of the term "modified" is specifically recited to relate back to the polypeptide. Accordingly, no impermissible new matter has been added by this claim amendment.

III. Response to Objections to the Claims

Claim 4 has been objected to as being improper for being awkward by not using the term "modified" such that it specifically relates back to the term "polypeptide". Claim 4 is herein amended such that the use of the term "modified" is specifically recited to relate back to the polypeptide. Accordingly, the objection has been obviated by amendment.

IV. Response to the Rejections under 35 U.S.C. § 112, Second Paragraph.

Claims 2-4 are rejected under 35 USC § 112, second paragraph, for allegedly failing to particularly point out and distinctly claim the subject matter. In response, claim 3 has been amended to recite "the polypeptide is a human heparan sulfate 3-*O*-sulfotransferase 5 polypeptide" rather than the polypeptide "comprises" a human heparan sulfate 3-*O*-sulfotransferase 5 polypeptide.

With respect to claim 2, Applicants respectfully disagree with the rejection and submit that the original claim is not unclear, as it is accurately written in a standard alternative drafting format easily understood by one of skill in the art. However, in order to facilitate prosecution of the application, claim 2 is herein amended to a Markush-type format thereby rendering this rejection moot. Applicants note for the record that no subject matter has been surrendered by the amendment of claim 2 from an alternative format to Markush-type format. With respect to claim 4, Applicants respectfully disagree with the rejection and submit that the original claim is not unclear, as one of ordinary skill in the art would understand what is meant by "modified to be in detectably labeled form". For example, it is standard procedure in the art to label a polypeptide to facilitate detection, such as by any number of procedures including, but not limited to, modification with affinity, fluorescent or radioactive labels. Applicants respectfully submit that one of ordinary skill in the art would understand what is meant by claim 4 and the claim is therefore not unclear.

Accordingly, Applicants respectfully submit that the § 112, second paragraph, rejections of claims 2-4 have been overcome and request withdrawal of the rejection of claims 2-4, as amended.

V. Response to the Rejections under 35 U.S.C. § 112, First Paragraph

Claims 1-4 are rejected under 35 USC § 112, first paragraph, for allegedly failing to comply with the written description and enablement requirements. Although Applicants believe the claims are fully enabled and have adequate written description, in order to expedite prosecution of the pending application, claims 1-4 are amended herein.

With respect to amended claims 1-4, Applicants respectfully assert that for the reasons described in detail below, one of ordinary skill in the art at the time of filing of the priority application would have recognized that Applicants were in possession of the isolated biologically active 3-O-sulfotransferase 5 (3-OST-5) polypeptides of amended claims 1-4. The instant specification allows a person of ordinary skill in the art to recognize the polypeptides that are being claimed, and recognition of what is being claimed suffices for compliance with the written description requirement. Therefore, Applicants respectfully assert that amended claims 1-4 are adequately supported by the instant application, and respectfully request that the rejection under 35 USC § 112, first paragraph, be withdrawn.

To satisfy the written description requirement, the applicant must convey to the skilled artisan that, as of the filing date sought, the applicant was in possession of the invention. *See Falkner* v. *Inglis,* 448 F.3d 1357,79 u.S.P.Q.2d 1001, 1007 (Fed. Cir. 2006) (citing *Vas-Cath, Inc.* v. *Mahurkar,* 935 F.2d 1555, 1563-64, 19 U.S.P.Q.2d 1111 (Fed. Cir. 1991). Applicants provide a full description of the 3-OST-5 polypeptide of SEQ ID NO 2, conveying to the skilled artisan that they were in possession of the 3-OST-5 polypeptide of SEQ ID NO 2. Further, Applicants also convey with this information that they were in possession of *all nucleic acids encoding* the 3-OST-5 polypeptide of SEQ ID NO 2. *See In re Wallach,* 71 U.S.P.Q.2d 1939 (Fed. Cir. 2004) (holding that the state of the art in molecular

biology has developed such that a complete amino acid sequence of a particular protein puts an inventor in possession of a genus of DNA sequences encoding it).

Further, Applicants convey to the skilled artisan that they were in possession of a genus of biologically active 3-OST-5 polypeptides having greater than 95% sequence identity to SEQ ID NO 2, encoded by a nucleic acid sequence having greater than 95% sequence identity to SEQ ID NO 1 or encoded by a nucleic acid molecule capable of hybridizing under stringent conditions to a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO 1, or a complement thereof. The Patent Office contends that a genus may be described by reference to a representative number of species within the genus sufficient to reasonably convey to the skilled artisan that Applicants were in possession of the claimed invention. *See* Official Action, page 5. The Patent Office further contends that the representative number of species can be sufficiently described by other relevant identifying characteristics, specific features and functional attributes that would distinguish different members of the claimed genus. *See* Official Action, page 5. Applicants provide such relevant identifying characteristics, specific features and functional attributes that distinguish different members of the claimed genus of biologically active 3-OST-5 polypeptides.

For example, Applicants provide a detailed description of the functional characteristics of 3-OST-5 polypeptides. This is in contrast to the allegation by the Patent Office at page 6 of the Official Action that the specification fails to demonstrate an assay specific for 3-OST-5 activity. Functional assays for 3-OST-5 polypeptide activity are provided in the specification at the Examples in the form of details for determining the substrate specificity and biological activities of 3-OST-5 isozymes. The Examples describe the differences in activity between the 3-OST-1, 3-OST-3 and 3-OST-5 isozymes. For example, the differences in binding to HSV-1 glycoprotein D (gD) and antithrombin (AT) of heparin sulfate (HS) modified by each of the 3-OST-1, 3-OST-3 and 3-OST-5 isozymes are shown in Tables 3 and 7. The differences in the HS products resulting from enzymatic modification by each of the 3-OST-1, 3-OST-3 and 3-OST-5 isozymes and the differences in biological activity of the various products formed are shown in Table 4. Accordingly, the specification

provides assays for selectively measuring 3-OST-5 isozyme activity, for example, by measuring the HS products formed by 3-OST-5 (3-OST-5 produces IdoUA2S-AnMan3S, GlcUA-AnMan3S6S, and IdoUA2S-AnMan3S6S whereas 3-OST-3 produces IdoUA2S-AnMan3S and IdoUA2S-AnMan3S6S and 3-OST-1 produces GlcUA-AnMan3S6S) or by measuring the binding of HS modified by 3-OST-5 to AT and gD (3-OST-5-modified HS binds to AT and gD whereas 3-OST-1-modified HS binds preferably to AT and 3-OST-3-modified HS binds preferably to gD). *See, Instant Specification*, pages 16-17, lines 28-32 and 1-10, respectively. Therefore, the assays for measuring function would have allowed a person of ordinary skill in the art to recognize the genus of polypeptides being claimed, and recognition of what is being claimed suffices for compliance with the written description requirement.

In addition to measurable functional characteristics, Applicants provide identifying structural characteristics of the genus of biologically active 3-OST-5 isozymes. Specifically, Applicants provide that the genus of polypeptides have greater than 95% sequence identity with SEQ ID NO 2, be encoded by a nucleic acid sequence having greater than 95% sequence identity to SEQ ID NO 1 or be encoded by a nucleic acid molecule capable of hybridizing under stringent conditions to a nucleic acid molecule of SEQ ID NO 1. The Federal Circuit in *Enzo Biochem Inc.* v. *Gen-Probe Inc.,* 296 F.3d 1316,63 U.S.P.Q.2d 1609 (Fed. Cir. 2002) held that a description *of functional characteristics of a claimed compound, coupled with a known or disclosed correlation between function and structure,* may adequately describe a claimed invention. *See Enzo, 63* U.S.P.Q.2d at 1613 (emphasis in original); *cf In re Robins,* 429 F.2d 452,166 U.S.P.Q. 552, 555 (C.C.P.A. 1970) ("Mention of representative compounds encompassed by generic claim language clearly is not required by §112 or any other provision of the statute."). The *Enzo* Court specifically stated that a compound may be adequately described by "stringent hybridization" to a known sequence because "such conditions dictate that all species within the genus will be structurally similar." *Enzo,* 63 U.S.P.Q.2d at 1615. Applicants provide such a description of the structural similarity of 3-OST-5 polypeptides coupled with functional characteristics and a known correlation between function and structure.

Applying the *Enzo* principles to the present case, the claimed genus of biologically active 3-OST-5 polypeptides are structurally related. Just like nucleic acids that hybridize under high stringency to a known sequence are structurally similar (see *Enzo* above); it reasonably follows that the proteins encoded by the nucleic acids are also structurally related. Likewise, polypeptides having greater than 95% sequence identity to a known sequence are highly structurally related to the known sequence. Therefore, the claimed genus of 3-OST-5 polypeptides having greater than 95% sequence identity to SEQ ID NO 2, encoded by a nucleic acid having greater than 95% sequence identity to SEQ ID NO 1 or encoded by a nucleic acid molecule capable of hybridizing under stringent conditions to a nucleic acid molecule of SEQ ID NO 1 are structurally similar.

In view of the known correlation between protein structure and function, it reasonably follows that structurally similar proteins are also functionally related. The protein structure-function relationship can be illustrated by the 3-OST isozymes in the instant case. For example, the 3-OST-1, 3-OST-3 and 3-OST-5 isozymes have similar activities in that they all attach sulfate to heparin sulfate. However, the isozymes show differences in substrate preference, which manifests in different biological functions. *See,* for example, the specification at pages 16-17, lines 28-32 and 1-10, respectively. The 3-OST-1 and 3-OST-3 isozymes are 72% and 58% identical, respectively, to the 3-OST-5 isozyme in the sulfotransferase domain. *See, Instant Specification*, page 16, lines 14-16. Given that the 3-OST-1 and 3-OST-3 isozymes have similar activity to 3-OST-5 but a relatively low degree of sequence identity (72% and 58%, respectively), the claimed genus of polypeptides having greater than 95% sequence identity to 3-OST-5 can be expected to have even greater functional similarity.

In light of the foregoing, the claimed genus of biologically active 3-OST-5 polypeptides is adequately described by reference to the single example of the protein of SEQ ID NO 2. Applicants provide measurable identifying functional characteristics of the claimed genus of biologically active 3-OST-5 polypeptides, the claimed genus of polypeptides is structurally similar to the 3-OST-5 polypeptide of SEQ ID NO 2, and there is a known correlation between polypeptide structure and

function. Therefore, Applicants have provided sufficient written description of the claimed genus of biologically active 3-OST-5 polypeptides and the rejection under 35 U.S.C. § 112, first paragraph, on the basis of a lack of written description, should be withdrawn.

Applicants further submit that the instant rejection of a genus of highly structurally related polypeptides where there is a disclosed assay for measuring function is believed to be in conflict with at least one non-binding decision of the Board of Patent Appeals and Interferences, *Ex parte Jon Elliot Adler*, Appeal No. 2006-0157 (2006) (hereinafter "*Adler*"; **Exhibit A**). One question at issue in the *Adler* case was whether a claimed genus of nucleotide sequences encoding bitter taste receptors met the requirements of 35 USC § 112, first paragraph. The Board of Patent Appeals and Interferences in *Adler* held as being adequately described and enabled, claims to nucleotide sequences encoding bitter taste receptor polypeptides having at least 95% sequence identity to a specific bitter taste receptor polypeptide or that hybridize under stringent conditions to a specific nucleotide sequence encoding the bitter taste receptor.

In the *Adler* case, the Examiner's rejection under 35 U.S.C. § 112, first paragraph, was stated as follows: "the claims encompass polynucleotides not described in the specification, e.g., mutated sequences, allelic variants, or sequences that have a recited degree of identity. None of these sequences meet the written description provision of 35 U.S.C. § 112, first paragraph." In its response, the court cited *Enzo* for the proposition that "the written description can be met by showing . . . relevant identifying characteristics . . . i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics." *Ex parte Jon Elliot Adler* citing *Enzo*, 63 U.S.P.Q.2d at 1613 (emphasis omitted). In its holding, the Board of Patent Appeals and Interferences stated: "The claims are limited to nucleic acids that hybridize under stringent conditions to SEQ ID NO:7 or that encode polypeptides at least 95% identical to SEQ ID NO:8. Thus, the claimed nucleic acids will necessarily have a high degree of structural similarity to SEQ ID NO:7 . . .". The court went on further to say that while

the specification did not allow those skilled in the art to know without testing which of the hybridizing or 95% identical sequences would encode polypeptides with the receptor function, the disclosure in the specification of an assay that could be used by one skilled in the art to determine function was sufficient written description for the claims to be found allowable.

The facts in the instant application are very close to those in *Adler*. The claims are similarly drawn to 3-OST-5 polypeptides having greater than 95% sequence identity to a specific 3-OST-5 polypeptide sequence, and an assay is provided in the instant specification for detecting 3-OST-5 activity. Applicants provide measurable identifying functional characteristics of the claimed genus of biologically active 3-OST-5 polypeptides, the claimed genus of polypeptides is structurally similar to the 3-OST-5 polypeptide of SEQ ID NO 2, and there is a known correlation between polypeptide structure and function. Therefore, the genus of biologically active 3-OST-5 polypeptides encompassed by amended claims 1-4 is adequately described by reference to the specific protein of SEQ ID NO 2. *See Enzo,* 63 U.S.P.Q.2d at 1615. The Patent Office has not shouldered its burden of presenting evidence to the contrary. *See In re Detiker,* 977 F.2d 1443, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992). The elements of a high level of structural similarity and assays for measuring function would have allowed a person of ordinary skill in the art to recognize the genus of polypeptides being claimed, and recognition of what is being claimed suffices for compliance with the written description requirement. Accordingly, Applicants have provided sufficient written description of the claimed genus of biologically active 3-OST-5 polypeptides and respectfully request withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, on the basis of a lack of written description.

Applicants similarly disagree with the Patent Office's rejection of claims 1-4 on the basis of a lack of enablement under 35 U.S.C. § 112, first paragraph. The Patent Office appears to be alleging that the specification enables only a nucleic acid encoding the 3-OST-5 polypeptide of SEQ ID NO 2. Applicants respectfully submit that the specification enables at least the genus of biologically active and structurally related 3-OST polypeptides of amended claims 1-4. Enablement is determined by

weighing several factors to determine whether undue experimentation would be required to make or use the claimed invention. *See In re Wands,* 858 F.2d 731, 736; 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). The amount of experimentation to practice the full scope of the claimed invention is not undue, if the experimentation is routine in nature and the techniques necessary to perform the experimentation are well known to the skilled artisan. *See, e.g., Johns Hopkins Univ.* v. *Cellpro, Inc.,* 152 F.3d 1342, 1360; 47 U.S.P.Q.2d 1705, 1719 (Fed. Cir. 1998) ("test [for undue experimentation] is not merely quantitative ... if it is merely routine"); *Falkner* v. *Inglis,* 448 F.3d 1357; 79 U.S.P.Q.2d 1001 (Fed. Cir. 2006) ("The person of ordinary skill in the art would clearly have possessed such knowledge, and given the ready accessibility of the journals, the absence of incorporation by reference is not problematic."); *Hybritech Inc.* v. *Monoclonal Antibodies, Inc.,* 802 F.2d 1367, 1384; 231 U.S.P.Q. 81, 94 (Fed. Cir. 1986) (A "patent need not teach, and preferably omits, what is well known in the art."). As provided in *In re Wands*, the experimentation required to make and use the genus of biologically active 3-OST-5 polypeptides of amended claims 1-4 is not undue, as it is routine in nature. Further, required techniques are well known to the skilled artisan, and/or are disclosed in the instant application.

For example, in the present case, undue experimentation would not be required to make and use a 3-OST-5 polypeptide having greater than 95% sequence identity to SEQ ID NO 2 or encoded by a nucleic acid molecule capable of hybridizing under stringent conditions to a nucleic acid molecule of SEQ ID NO 1, because amino acid alterations can be made to the proteins having the sequence of SEQ ID NO 2 by standard molecular biological procedures including, for example, oligonucleotide-directed mutagenesis, and procedures for performing stringent hybridizations are well known in the art. The present disclosure provides the complete sequence of nucleic acids encoding the protein of SEQ ID NO 2, so the experimentation required to make a mutation to the encoding nucleic acids is routine. In addition, Applicants provide assays to determine whether the modified protein possesses 3-OST-5 activity. *See, e.g., Instant Specification*, Examples 2-9. It would be routine for one of ordinary skill in the art to perform the detailed assays provided in

the specification for measuring 3-OST-5 activity. Further, guidance to make the appropriate amino acid modifications is provided by the sequence comparison in Figure 2 showing conserved and less conserved regions between the 3-OST isozymes, 3-OST-1, 3-OST-3A, 3-OST-3B and 3-OST-5.

The skilled artisan further appreciates that limited amino acid alterations, *e.g.*, a single amino acid modification, *generally* can be made with a reasonable expectation of maintaining protein function. Gassner *et al., Proc. Nat'l Acad. Sci USA* 93: 12155-58 (1996); (hereinafter "*Gassner et al.*"; (**Appendix A**)) reveals that considerable (up to 10) amino acid alterations can be made even to the tightly packed core of a globular protein without eliminating activity or folding of the protein. Wells, *Biochemistry* 29: 8509-17 (1990) (hereinafter "*Wells*"; (**Appendix B**)) discloses that the free energy changes in mutant proteins *generally* are additive with increasing numbers of amino acid mutations. *Wells* shows that proteins generally function when they have single, or even multiple, amino acid changes. *Wells* states: "[R]emoval of a single molecular contact by a point mutation causes relatively small reductions (typically 0.5-5 kcal/mol) in the free energy of transition-state destabilization, protein-protein interactions, or protein stability compared to the overall free energy associated with these functional properties (usually 5-20 kcal/mol)." *Wells*, page 8509, left col. (citations omitted). This means that a protein typically can be modified at one or several amino acid positions without loss of its functional properties. *Wells* also concludes that the effect of most mutations on structure is highly localized, and thus less likely to affect function. *Wells*, page 8516, left col.

In the present case, as evidenced by *Gassner et al.* and *Wells*, for example, the proteins with altered sequences that are encompassed by amended claims 1-4 would be reasonably expected to retain biological function. In any case, the variant polypeptides without biological activity could be identified with routine experimentation. 35 U.S.C. § 112, first paragraph, does not require disclosure of a test with *every* species covered by a claim, even in an unpredictable art. *In re Angstadt,* 537 F.2d 498, 502, 190 U.S.P.Q. 214, 218 (C.C.P.A. 1976). Further, some experimentation to determine which embodiments encompassed by the claims will work is permitted without the experimentation being undue in nature. *See Angstadt,*

190 U.S.P.Q. at 218; *Wands,* 858 F.2d 736-37 ("Enablement is not precluded by the necessity for some experimentation such as routine screening."). Accordingly, the experimentation required to make and use the genus of biologically active 3-OST-5 polypeptides of amended claims 1-4 is not undue, as it is routine in nature and the required techniques are well known to the skilled artisan.

Furthermore, the routine nature of the experimentation required to practice the full scope of the instant claims is supported by the holding in *Alder.* As described above for written description, in *Adler* the court held as being adequately described and enabled, claims to nucleotide sequences encoding bitter taste receptor polypeptides having at least 95% sequence identity to a specific bitter taste receptor polypeptide or that hybridize under stringent conditions to a specific nucleotide sequence encoding the bitter taste receptor. On facts very similar to those in the instant case, the court in *Adler* found the Examiner had not adequately explained why practicing the full scope of the claims would have required undue experimentation. The *Adler* court found that undue experimentation would not be required to make and use the claimed genus of bitter taste receptors. In its decision, the court stated that the specification provides the nucleotide and amino acid sequences for the human bitter taste receptor. The court went on to say that the specification also discloses an amino acid sequence comparison of twenty three human, mouse and rat bitter taste receptors that identifies conserved and less conserved regions, thereby providing guidance to those skilled in the art regarding what regions are likely to be required for function. According to the *Adler* court, it is known in the art that changes in conserved regions are more likely to disrupt function of the protein than changes in non-conserved regions. Thus, the court stated that the *Adler* specification guides a skilled worker to areas of the bitter taste receptors that are likely to be tolerant to amino acid changes.

The court in *Adler* also pointed out that the specification discloses assays for determining whether a particular bitter taste receptor retains the activity of the wild-type protein. In its holding, the court based its finding that the Examiner failed to show nonenablement by a preponderance of evidence on 1) the prior art providing substantial guidance with respect to the direction the experimentation should

proceed; 2) a process being provided for making and assaying the mutated proteins (noting the process "to be routine, if tedious, experimentation"); and 3) the claimed genus being limited to variants having 95% or less variation compared to the wild-type sequence or hybridizing under stringent conditions to the nucleic acid encoding the wild-type protein.

Similar to the *Adler* case, the instant specification provides the nucleotide and amino acid sequences shown in SEQ ID NOs 1 and 2, corresponding to human 3-OST-5. The instant specification also discloses an amino acid sequence comparison of the 3-OST-1, 3-OST-3A, 3-OST-3B and 3-OST-5 isozymes showing conserved and less conserved regions, thereby providing guidance to those skilled in the art regarding what regions are likely to be required for function and less tolerant to amino acid changes. *See* Figure 2. Like in *Adler*, the instant specification discloses assays for determining whether a particular 3-OST-5 polypeptide retains the activity of the wild-type protein. *See* Examples 1-9. Accordingly, like in the *Adler* case, the experimentation required to make and use the genus of biologically active 3-OST-5 polypeptides of amended claims 1-4 is not undue, as it is routine in nature and the required techniques are well known to the skilled artisan. Therefore, amended claims 1-4 are sufficiently enabled and comply with the requirement of 35 U.S.C. § 112, first paragraph. Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 112, first paragraph, on the basis of a lack of enablement.

CONCLUSION

In light of the above amendments and remarks, it is respectfully submitted that the present application is now in proper condition for allowance, and an early notice to such effect is earnestly solicited.

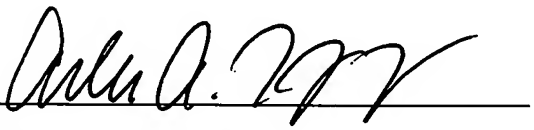
If any small matter should remain outstanding after the Patent Examiner has had an opportunity to review the above Remarks, the Patent Examiner is respectfully requested to telephone the undersigned patent attorney in order to resolve these matters and avoid the issuance of another Official Action.

DEPOSIT ACCOUNT

The Commissioner is hereby authorized to charge any deficiencies of payment or credit any overpayment associated with the filing of this correspondence to Deposit Account No. **50-0426**.

Respectfully submitted,

JENKINS, WILSON, TAYLOR & HUNT, P.A.

Date: January 14, 2008 By: 

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421/67 PCT/US AAT/LLK/dbp

Exhibit A

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte JON ELLIOT ADLER

Appeal No. 2006-0157
Application No. 09/825,882

HEARD: March 23, 2006

Before SCHEINER, MILLS, and GRIMES, Administrative Patent Judges.

GRIMES, Administrative Patent Judge.

DECISION ON APPEAL

This appeal involves claims to nucleic acids encoding a bitter taste receptor. The examiner has rejected the claims as lacking utility, non-enabled, and inadequately described. We have jurisdiction under 35 U.S.C. § 134. We reverse all of the rejections.

Exhibit A

Background

"Mammals are believed to have five basic taste modalities: sweet, bitter, sour, salty, and umami (the taste of monosodium glutamate)." Specification, page 1. "Each taste modality is believed to be mediated by distinct transduction pathways. These pathways are believed to be mediated by receptors . . . expressed in subsets of taste receptor cells." Id.

The specification discloses a "family of G Protein-Coupled Receptors . . . thought to be primarily involved in bitter taste transduction." Page 4. The family of proteins is known as the "T2R" family; the nucleic acid sequence of human T2R61 is shown in the specification's SEQ ID NO:7 and the encoded amino acid sequence is shown in SEQ ID NO:8.

The specification discloses that T2R proteins are useful for, among other things, "screening for modulators, e.g., activators, inhibitors, stimulators, agonist, and antagonists, of these novel taste-cell-specific GPCRs. . . . These methods of screening can be used to identify high affinity agonists and antagonists of taste cell activity. These modulatory compounds can then be used in the food and pharmaceutical industries to customize taste, for example, to decrease or mask the bitter taste of food or drugs." Page 9. See also page 3: "Such taste modulating compounds could be useful in the pharmaceutical and food industries to improve the taste of a variety of consumer products, or to block undesirable tastes, e.g., bitter tastes, in certain products."

Discussion

1. Claim construction

Claims 158-185 are pending and on appeal. Claims 158 and 159 are representative and read as follows:

158. An isolated nucleic acid molecule encoding a bitter taste receptor selected from the group consisting of

(i) an isolated nucleic acid sequence having the nucleic acid sequence contained in SEQ ID NO:7;

(ii) a nucleic acid sequence that encodes the bitter taste polypeptide contained in SEQ ID NO:8;

(iii) an isolated DNA sequence that hybridizes under stringent hybridization conditions to the nucleic acid sequence contained in SEQ ID NO:7 wherein stringent hybridization conditions are hybridization in 5 x SSC, 1% SDS, incubated at 65°C and wash in 0.2 x 55C and 0.1% SDS at 65°C, wherein said hybridization and wash steps are each effected for at least 1 minute.

159. An isolated nucleic acid molecule encoding a bitter taste receptor polypeptide which polypeptide comprises at least 95% identity to the taste receptor polypeptide contained in SEQ ID NO:8, wherein sequence identity is determined by any on of the BLAST, BLASST 2.0 or PILE UP algorithms.

Thus, claim 158 is directed to SEQ ID NO:7, another nucleic acid that encodes the amino acid sequence of SEQ ID NO:8, or a DNA sequence that hybridizes under specified, stringent conditions to SEQ ID NO:7. Claim 159 is directed to a nucleic acid that is at least 95% identical to SEQ ID NO:7. Both claims also require that the nucleic acids encode functional bitter taste receptors.

2. Utility

The examiner rejected claims 158-185 under 35 U.S.C. §§ 101 and 112, first paragraph, on the basis that the specification does not disclose a patentable utility for the claimed nucleic acids. The examiner reasoned that

> [t]he concept of "bitter taste" is known to involve multiple and as yet poorly characterized transduction schemes. . . . These transduction schemes are also thought to involve a large diversity of receptors. . . . The specification has given no indication as to which of these [bitter-tasting] compounds is expected to bind to and activate SEQ ID NO:8. Without such knowledge, the artisan could not use the protein to manipulate any aspect of the senses involving taste.

Examiner's Answer, pages 4-5. The examiner acknowledged that the "specification puts forth that the polypeptides are useful for 'representing the perception of taste and/or for predicting the perception of taste in a mammal'," "as probes to dissect taste-induced behaviors," and "in a screening method to determine what molecules may

activate or inhibit the polypeptides," but concluded that "[t]hese proposed uses lack a substantial utility, because each of the proposed uses are of a general nature." Id., page 5.

Appellant argues that the evidence of record supports the specification's assertion that SEQ ID NO:8 is a bitter taste receptor. See the Appeal Brief, pages 11-15. Appellant also argues that "most significantly, and as correctly disclosed in the as-filed application, the subject T2R nucleic acid sequences, based in their reasonably anticipated . . . functionality as bitter taste receptors can be used in high throughput screens to identi[f]y compounds which modulate the activity of this receptor." Id., page 20.

The U.S. Court of Appeals for the Federal Circuit recently addressed the utility requirement in the context of a claim to DNA. See In re Fisher, 421 F.3d 1365, 1371, 76 USPQ2d 1225, 1229 (Fed. Cir. 2005). The Fisher court held that § 101 requires a utility that is both substantial and specific. Id. at 1371, 76 USPQ2d at 1229. A substantial utility is one that "show[s] that an invention is useful to the public as disclosed in its current form, not that it may be useful at some future date after further research. Simply put, to satisfy the 'substantial' utility requirement, an asserted use must show that that claimed invention has a significant and presently available benefit to the public." Id., 76 USPQ2d at 1230.

A specific utility is one " which is not so vague as to be meaningless." Id. In other words, "in addition to providing a 'substantial' utility, an asserted use must show that that claimed invention can be used to provide a well-defined and particular benefit to the public." Id.

When we apply the standards set out in Fisher to the facts of this case, we conclude that the examiner's rejection must be reversed. As we understand it, the examiner does not dispute that the protein encoded by the claimed nucleic acids is a bitter taste receptor, but contends that those skilled in the art could not use the protein for any specific and substantial way without first knowing what compounds were bound by it.

We find this position untenable. The specification discloses that the T2R61 protein can be used in screening assays to identify compounds that bind the protein. Given the undisputed characterization of the protein as a bitter taste receptor, it seems reasonable to expect that some of those compounds would be antagonists that would block the perception of bitter taste by the receptor. As Chandrashekar[1] put it, "the identification of human bitter taste receptors makes it possible to use high-throughput screening strategies to identify bitter taste antagonists, and in a small but significant way, eliminate bitterness from the world." Page 710.

This utility is disclosed in the specification (see page 9) and relied on in the Appeal Brief (see page 20). It also seems to us to be a "substantial" and "specific" utility, as defined by the Fisher court: identifying compounds that can block the bitter taste of foods or medicines would seem to provide a significant, presently available, and well-defined benefit to the public.

The examiner bears the initial burden of showing that a claimed invention lacks patentable utility. See In re Brana, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995). In this case, however, the examiner's explanation of the rejection (Examiner's Answer, pages 4-8) does not address this asserted utility. The examiner's reasoning does not persuade us that the disclosed utility is inadequate to meet the requirements of 35 U.S.C. § 101. We therefore reverse the rejections under 35 U.S.C. §§ 101 and 112, first paragraph, based on lack of utility.

[1] Chandrashekar et al., "T2Rs function as bitter taste receptors," Cell, Vol. 100, pp. 703-711 (2000). Chandrashekar was incorporated by reference into the specification. See page 8, lines 14-15.

3. Scope of enablement

The examiner also rejected claims 158, 159, and 164-185 under 35 U.S.C. § 112, first paragraph, on the basis that "the specification does not enable any person skilled in the art to make and use the invention commensurate in scope with these claims." Examiner's Answer, page 8. The examiner noted that these claims "encompass polynucleotides encoding polypeptide variants of the polypeptide of SEQ ID NO:8[;] i.e., substitutions, deletions or insertions." Id., pages 8-9. The examiner reasoned "the specification has failed to teach one of ordinary skill in the art which amino acid substitutions, deletions or insertions to make that will preserve the structure and function of the protein, that "no particular ligand has been disclosed to bind and activate the protein, so the artisan would not know how to test variants for functionality," that the specification provides no working examples of T2R61 variants, and that the prior art (including Chandrashekar) "recognizes the complexity, unpredictability, and non-routine nature of the work involved in trying to assay functional T2R receptors. Id., pages 9-11. The examiner concluded that undue experimentation would be required to make and use the claimed variants.

Appellant argues that the claimed genera are limited to nucleic acids with a high degree of similarity to SEQ ID NO:7 or encoding a protein very similar to SEQ ID NO:8, that such can be made or isolated routinely, and that the specification "provides substantial information relating to T2R assays that would enable one skilled in the art to screen these variant hT2R61 nucleic acid sequences and identi[f]y those variants that are functional, i.e., bind the same bitter ligands which specifically interact with wild-type hT2R61 polypeptide." Appeal Brief, pages 25-27.

"[T]o be enabling, the specification of a patent must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation.'" In re Wright, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). "That some experimentation may be required is not fatal; the issue is whether the amount of experimentation required is 'undue.'" In re Vaeck, 947 F.2d 488, 495, 20 USPQ2d 1438, 1444 (Fed. Cir. 1991). Whether the amount of experimentation required is undue is determined by reference to the well-known Wands factors. See In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).

In this case, we agree with Appellant that the examiner has not shown, by a preponderance of the evidence, that practicing the full scope of the claims would have required undue experimentation. The examiner's position rests in part on the lack of guidance in the specification regarding amino acids in T2R61 can be altered or deleted without affecting its function.

It is true that the specification does not teach, in so many words, which amino acids are critical to T2R61 function. However, the specification does provide substantial guidance to those skilled in the art. The specification provides the nucleotide and amino acid sequences shown in SEQ ID Nos:7 and 8, which correspond to human T2R61. The specification also incorporates by reference the sequence comparison disclosed by Adler.[2] Adler provides a sequence comparison of twenty-three human, mouse, and rat T2R amino acid sequences. The comparison does not include human T2R61 but the examiner has given us no reason to think that a person skilled in the art could not apply the same techniques used by Adler to the T2R61 sequence disclosed in the instant specification.

Adler's sequence comparison identifies each of the seven transmembrane (TM) domains, and shows conserved regions between TM1 and TM2, between TM3 and TM4, and between TM5 and TM6. By comparison, the regions between TM2 and TM3, between TM4 and TM5, and between TM6 and TM7 are much less conserved. Such

[2] Adler et al., "A novel family of mammalian taste receptors," Cell, Vol. 100, pp. 693-702 (2000). Adler was incorporated by reference into the specification. See page 8, lines 13-15.

sequence comparisons provide guidance to those skilled in the art regarding what regions of the T2R proteins are likely to be required for function: changes in conserved regions are more likely to disrupt function of the protein than changes in non-conserved regions. Thus, Adler guides a skilled worker to areas of T2R61 that are likely to be tolerant of amino acid changes.

The specification also discloses assays for determining whether a T2R61 variant retains the activity of the wild-type protein. See pages 50-65 (discussing numerous assays to test for binding of a T2R receptor to putative taste modifiers). Chandrashekar provides evidence that such assays identify functional bitter taste receptors. See the abstract: "[W]e use a heterologous expression system to show that specific T2Rs function as bitter taste receptors. A mouse T2R (mT2R-5) responds to the bitter tastant cycloheximide, and a human and a mouse receptor (hT2R-4 and mT2R-8) responded to denatonium and 6-n-propyl-2-thiouracil."

Finally, Appellant has presented post-filing evidence, which the examiner has considered (Examiner's Answer, page 17), that confirm that the methods disclosed in the specification demonstrate that hT2R61 intereacts with several compounds that elicit a bitter taste. See the evidence attached to the Appeal Brief as Exhibit 3.

On the facts of this case, we must agree with Appellant that the examiner has not adequately explained why practicing the full scope of the claims would have required undue experimentation. The prior art, which was incorporated by reference into the specification, provides substantial guidance with respect to the direction the experimentation should proceed. The process of making and assaying mutated proteins would appear to be routine, if tedious, experimentation. The claims are limited to variants having 5% or less variation compared to SEQ ID NO:8 and those hybridizing to SEQ ID NO:7 under stringent conditions. In view of these factors, we cannot say that the examiner has shown nonenablement by a proponderance of the evidence. The rejection of claims 158, 159, and 164-185 for lack of enablement is reversed.

4. Written description

The examiner also rejected claims 158, 159, and 164-185 under 35 U.S.C. § 112, first paragraph, on the basis that "the claims encompass polynucleotides not described in the specification, e.g., mutated sequences, allelic variants, or sequences that have a recited degree of identity. None of these sequences meet the written description provision of 35 U.S.C. § 112, first paragraph." Examiner's Answer, page 12. The examiner reasoned that

> [t]he specification has not provided a particular essential feature, either a functional or structural feature, that the claimed genus of polynucleotides possess. The recitation of the property of hybridization does not, alone, provide sufficient information regarding the structure of the claimed polynucleotide variants. Further, most of these variants are expected to encode polypeptides having an amino acid sequence different than that of SEQ ID NO:8 and thus having different structural and functional properties.

The examiner "'bears the initial burden . . . of presenting a prima facie case of unpatentability.' In re Oetiker, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). Insofar as the written description requirement is concerned, that burden is discharged by 'presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims.'" In re Alton, 76 F.3d 1168, 1175, 37 USPQ2d 1578, 1583 (Fed. Cir. 1996).

Several recent decisions of the U.S. Court of Appeals for the Federal Circuit have addressed the written description of inventions involving DNA. In University of California v. Eli Lilly and Co., the court held that "[a]n adequate written description of a

DNA . . . 'requires a precise definition, such as by structure, formula, chemical name, or physical properties.'". 119 F.3d 1559, 1566, 43 USPQ2d 1398, 1404 (Fed. Cir. 1997). Where a genus of DNAs was claimed, as here, the Lilly court noted that those skilled in the art can "visualize or recognize the identity of the members of [a fully described] genus" and held that "[a] description of a genus of DNAs may be achieved by means of a recitation of a representative number of DNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to the members of the genus, which features constitute a substantial portion of the genus." Id. at 1568, 43 USPQ2d at 1406.

The court clarified the Eli Lilly standard in Enzo Biochem, Inc. v. Gen-Probe Inc., 323 F.3d 956, 964, 63 USPQ2d 1609, 1613 (Fed. Cir. 2002). The Enzo court adopted the standard that "the written description requirement can be met by 'show[ing] that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . . i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of such characteristics.'" Id. at 964, 63 USPQ2d at 1613 (emphasis omitted, bracketed material in original).

Finally, the court has made clear that other factors, including the level of skill in the art, are relevant to whether a description satisfies § 112. See Capon v. Eshhar, 418 F.3d 1349, 1358-59, 76 USPQ2d 1078, 1085 (Fed. Cir. 2005) ("Precedent illustrates that the determination of what is needed to support generic claims to biological subject matter depends on a variety of factors, such as the existing knowledge in the particular field, the extent and content of the prior art, the maturity of the science or technology, the predictability of the aspect at issue, and other considerations appropriate to the subject matter.").

When we apply the appropriate legal standard to the facts of this case, we conclude that the examiner's rejection must be reversed. The claims are limited to nucleic acids that hybridize under stringent conditions to SEQ ID NO:7 or that encode polypeptides at least 95% identical to SEQ ID NO:8. Thus, the claimed nucleic acids will necessarily have a high degree of structural similarity to SEQ ID NO:7; in other words, SEQ ID NO:7 shares something like 95% of its structure with each of the claimed nucleic acids.

Although the specification's disclosure does not allow those skilled in the art to know, without testing, which of the hybridizing or 95% similar sequences will encode a polypeptide that shares hT2R61's bitter taste receptor function, the specification teaches numerous assays that can be used to make that determination. See pages 50-65. The prior art Chandrashekar reference provides evidence that carrying out such assays was within the level of ordinary skill. See pages 703-707.

Because the claimed nucleic acids share a large proportion of their structure with SEQ ID NO:7 and because assays that are disclosed in the specification and apparently routine to those skilled in the art can be used to distinguish between functional and nonfunctional embodiments, we conclude that a person of ordinary skill in the art would have recognized from the specification's description that the inventors were in possession of the claimed nucleic acids. The rejection of claims 158, 159, and 164-185 for lack of adequate written description is reversed.

Summary

We conclude that the evidence of record does not support the rejections for lack of utility, nonenablement, and lack of adequate written description. The rejections on appeal are reversed.

REVERSED

TONI R. SCHEINER Administrative Patent Judge	)
DEMETRA J. MILLS Administrative Patent Judge	) BOARD OF PATENT) APPEALS AND) INTERFERENCES
ERIC GRIMES Administrative Patent Judge	)

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A test of the "jigsaw puzzle" model for protein folding by multiple methionine substitutions within the core of T4 lysozyme

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Contributed by Brian W. Matthews, August 14, 1996

ABSTRACT **To test whether the structure of a protein is determined in a manner akin to the assembly of a jigsaw puzzle, up to 10 adjacent residues within the core of T4 lysozyme were replaced by methionine. Such variants are active and fold cooperatively with progressively reduced stability. The structure of a seven-methionine variant has been shown, crystallographically, to be similar to wild type and to maintain a well ordered core. The interaction between the core residues is, therefore, not strictly comparable with the precise spatial complementarity of the pieces of a jigsaw puzzle. Rather, a certain amount of give and take in forming the core structure is permitted. A simplified hydrophobic core sequence, imposed without genetic selection or computer-based design, is sufficient to retain native properties in a globular protein.**

The cores of globular proteins consist of buried, primarily hydrophobic, amino acids. Tight packing of the amino acid side chains (1) has led to the idea that the size and shape of the nonpolar amino acids within the core may constrain or define the overall protein fold (2, 3). This "jigsaw puzzle" model of protein folding was originally introduced by Crick (4) as a "knobs into holes" description of α-helix packing and has been elaborated by Chothia *et al.* (5), and by Alber and co-workers (6). Here the jigsaw puzzle model refers to shape complementarity (3), not to the pathway of folding (7). The model is supported by the observation that changes in the sizes and shapes of residues within the cores of proteins are usually destabilizing (8–10). Also in support of the model, the structures of α-helical coiled coils appear to be determined by the shapes of the buried side chains (6). In contrast with this view, it has been shown that alternative core sequences that lead to viable proteins could be selected by random mutagenesis for both λ-repressor (11) and T4 lysozyme (12), among others (13, 14). It is possible, however, that a limited number of combinations of amino acids are viable and that they are the ones identified by the mutagenic selection. Here we explore an approach in which there is no selection other than the sites of substitution.

MATERIALS AND METHODS

We chose methionine as a generic core-replacement residue for a combination of reasons. First, a methionine side chain occupies roughly the same volume as the frequently observed core residues leucine, isoleucine, and phenylalanine. It is, however, more flexible and can more readily adapt to occupy whatever space might be available. In this sense methionine contrasts with the rigid, predetermined shape of a piece of a jigsaw puzzle. Methionine also occurs relatively infrequently in known proteins (15). Thus multiple methionine substitutions would be expected to substantially change the composition of the core. Finally, we wondered if the introduction of multiple, flexible, amino acids within the core of a protein might lead to the onset of molten globule characteristics (16).

Table 1. Activity and stability of methionine-substituted lysozymes

Mutant	Activity (%)	ΔT_m (°C)	$\Delta H(T_m)$ (kcal/mol)	ΔH(ref) (kcal/mol)	$\Delta\Delta G$ (kcal/mol)
WT*	100		130	115	—
I78M	70	−3.7	117	111	−1.5
L84M	104	−4.9	110	108	−1.9
L91M	96	−2.0	125	115	−0.8
L99M†	90	−1.3	134	122	−0.4
I100M	105	−4.5	125	121	−1.6
V103M	70	−3.1	117	109	−1.2
L118M	98	−1.8	130	119	−0.7
L121M	87	−2.1	129	119	−0.8
L133M	106	−1.0	128	115	−0.4
F153M†	87	−1.6	128	116	−0.6
7-Met‡	43	−14.5	96	117	−5.0
10-Met‡	≈20	−25	42	88	−7.3

The activity was determined as in ref. 28 except at 20°C in 66 mM potassium phosphate, pH 6.8. For the 10-Met mutant, a loss in activity was seen with time. Activities were also determined using lysis plates (29) and found to be in agreement with the values given in the table.

Stability measurements (18) were made in 0.1 M sodium chloride/1.4 mM acetic acid/8.6 mM sodium acetate, pH 5.42. The melting temperature, T_m, of WT* lysozyme was 65.3°C. ΔT_m is the change in the T_m of the mutant relative to wild type. For the single mutants, the uncertainty in ΔT_m os ±0.2°C; for the multiple mutants it is ±0.5°C. $\Delta H(T_m)$ is the enthalpy of unfolding measured at T_m. The uncertainty is ±5 kcal/mol. ΔH(ref) is the enthalpy of unfolding calculated at the reference temperature of 59°C using a constant ΔC_p of 2.5 kcal/mol·deg. $\Delta\Delta G$ is the free energy of unfolding of the mutant relative to wild type. ΔG values were computed at 59°C using a constant ΔC_p of 2.5 kcal/mol·deg. The uncertainty in $\Delta\Delta G$ is ±0.1 kcal/mol for the single mutants and ±0.4 kcal/mol for the 7-Met replacement. Because of the low value of ΔH of the 10-Met mutant, $\Delta\Delta G$ was determined at the T_m of the mutant with an estimated uncertainty of about 1 kcal/mol.

†Mutants L99M and F153M were described previously (18).

‡The 7-Methionine mutant includes the substitutions L84M/L91M/L99M/L118M/L121M/L133M/F153M. The 10-Methionine variant includes the additional substitutions I78M/I100M/V103M. The molecular masses of these proteins determined by mass spectrometry agreed with the theoretical values, suggesting little if any oxidization of the introduced methionines (data not shown).

All sites of substitution are buried within the carboxyl-terminal domain of T4 lysozyme, and the side chain of each residue contacts at least one other side chain of the set. The 10 single-site mutants as well as various multiple-methionine mutants were constructed (17) in cysteine-free pseudo wild-type lysozyme, hereafter identified as WT* or wild type (18). Activity and stability measurements for the 10 single mutants, together with the 7-Met and 10-Met mutants, are listed in Table 1.

RESULTS AND DISCUSSION

All variants possessed native-like properties. The thermal denaturations of the one- and seven-methionine variants are

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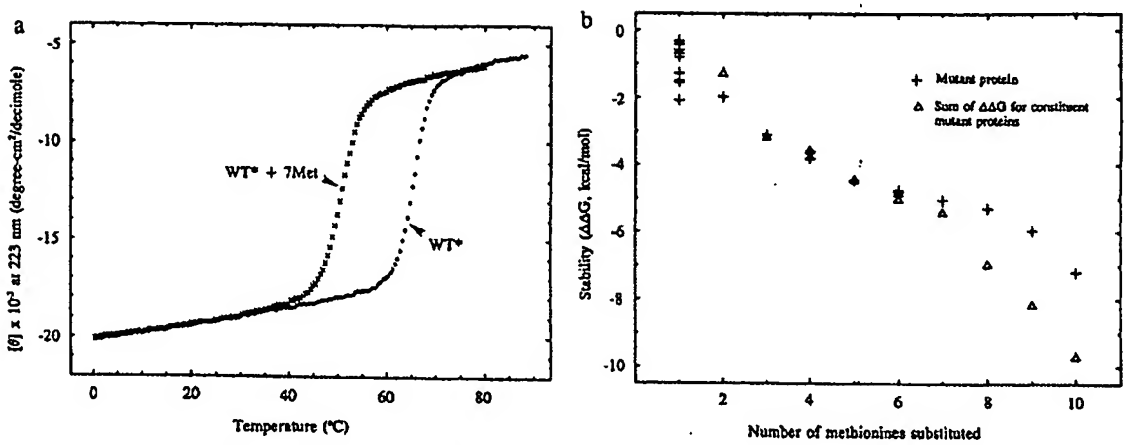

FIG. 1. (*a*) Comparison of the thermal unfolding transition of the seven-methionine mutant with that of wild-type lysozyme. (*b*) Stabilities for mutant lysozymes plotted as a function of the number of introduced methionines. The crosses show the stabilities, relative to wild-type, of the single mutants and the seven- and 10-methionine mutants listed in Table 1. Additional crosses show the stabilities of other multiple-methionine variants that have been constructed but are not described explicitly in Table 1 (unpublished results). The triangles show the sums of the stabilities of the single mutants that are combined together to obtain a given multiple mutant. Two different combinations of substitutions were used to obtain 4-Met and 6-Met lysozymes. The stabilities of both constructs are included in the figure.

essentially as cooperative as wild-type, with comparable enthalpies of unfolding (Fig. 1*a*; Table 1). The 10-methionine variant unfolds cooperatively, although the enthalpy is reduced (Table 1). Activity was equal to at least 20% that of wild type, suggesting that active site structure is retained (Table 1). The aromatic circular dichroism spectra of the largest construct, a 10-methionine core variant, was comparable in shape and magnitude to the spectra of wild type. The one-dimensional NMR spectrum of the same variant had significant chemical shift dispersion (data not shown). Taken together these results strongly suggest that the 10-Met variant has a well-defined three-dimensional structure and is not a molten globule (16).

Crystals of the 7-Met variant (Table 1) were obtained and found to be isomorphous with wild-type lysozyme (18). X-ray data to a 1.9-Å resolution, 92% complete, were measured at room temperature (19, 20). A difference density map (Fig. 2*a*) showed seven well defined positive peaks corresponding to the introduction of the electron-dense sulfur at each of the sites of substitution as well as negative density where atoms were deleted.

The variant structure (Fig. 3) was refined (21, 22) to a crystallographic residual of 15.2% with bond lengths and angles within 0.18 Å and 3.0° of ideal values and was found to be very similar to wild type. The coordinates have been deposited in the Brookhaven Data Bank. The root-mean-square discrepancy of the main chain atoms within the carboxyl-terminal domain is 0.20 Å. In the six cases in which a methionine replaced a leucine, the χ_1 and χ_2 values in the mutant were similar to those in wild type. Thus, each of the substituted methionines essentially traced the path of the residue that it replaced. For the Phe-153 → Met substitution, however, χ_1 changed by 92° to avoid a steric clash.

The crystallographic thermal factors of the side chains of the seven methionines are, on average, marginally less than the thermal factors of the amino acids that they replace (24.0 $Å^2$ versus 25.7 $Å^2$). The distal methyl groups are also well-ordered (average thermal factor 23.8 $Å^2$). As shown in Fig. 2*b*, the mobility of the surrounding side chains in the mutant structure is also very similar to wild type. Therefore there is no suggestion that the substitution of seven methionines leads to disorder of the hydrophobic core.

As more and more methionines are introduced into the protein, the overall stability decreases (Fig. 1; Table 1). When six or more methionines are substituted, the loss of stability is somewhat less than the sum of the constituent single replacements (Fig. 1*b*) with the discrepancy increasing to a maximum of 2.5 kcal/mol for the 10-methionine construct. This indicates that there is some relaxation in the polymethionine protein that either introduces new, favorable, interactions or relieves some of the strain associated with the single substitutions. The loss in protein stability is understandable. For each methionine replacement there is a reduction in the solvent transfer free energy (about 0.6 kcal/mol for Leu to Met) (23). Also the side chain of methionine has more degrees of freedom than do other hydrophobic core amino acids. Each methionine-to-leucine replacement at a restricted, internal, site is predicted to have an entropy cost of about 0.8 kcal/mol (24, 25). Taken together, these two factors are expected to reduce the stability of the seven-methionine mutant by about 10 kcal/mol relative to wild type. Some of the replacements may also decrease stability because of introduced strain. The actual loss in stability for the 7-Met mutant is only 5.0 kcal/mol, suggesting that the above estimate of ≈10 kcal/mol is too high.

The finding that 10 core residues in T4 lysozyme can be replaced with methionine supports the overall importance of the hydrophobic effect in protein folding. At the same time, the results show that the interaction between the core residues is not strictly comparable with the precise spatial complementarity of the pieces of a jigsaw puzzle. Rather, a certain amount of give and take in forming the core structure is permitted. This is in contrast to α-helical coiled-coils where changes in the shape of hydrophobic residues can lead to different packing arrangements (6).

The observation that methionines substituted at various internal sites remain well ordered suggests that selenomethionine, or telluro methionine, introduced in this fashion should be suitable for MAD phasing (26). The apparent lack of oxidation of core sites, presumably due to reduced oxygen accessibility, may aid in the use of such oxygen-sensitive analogs.

Previous studies have shown that multiple alanine replacements can be made on the surface of T4 lysozyme, at least within α-helices, with little change in structure or stability (27). The present study shows that multiple replacements with a

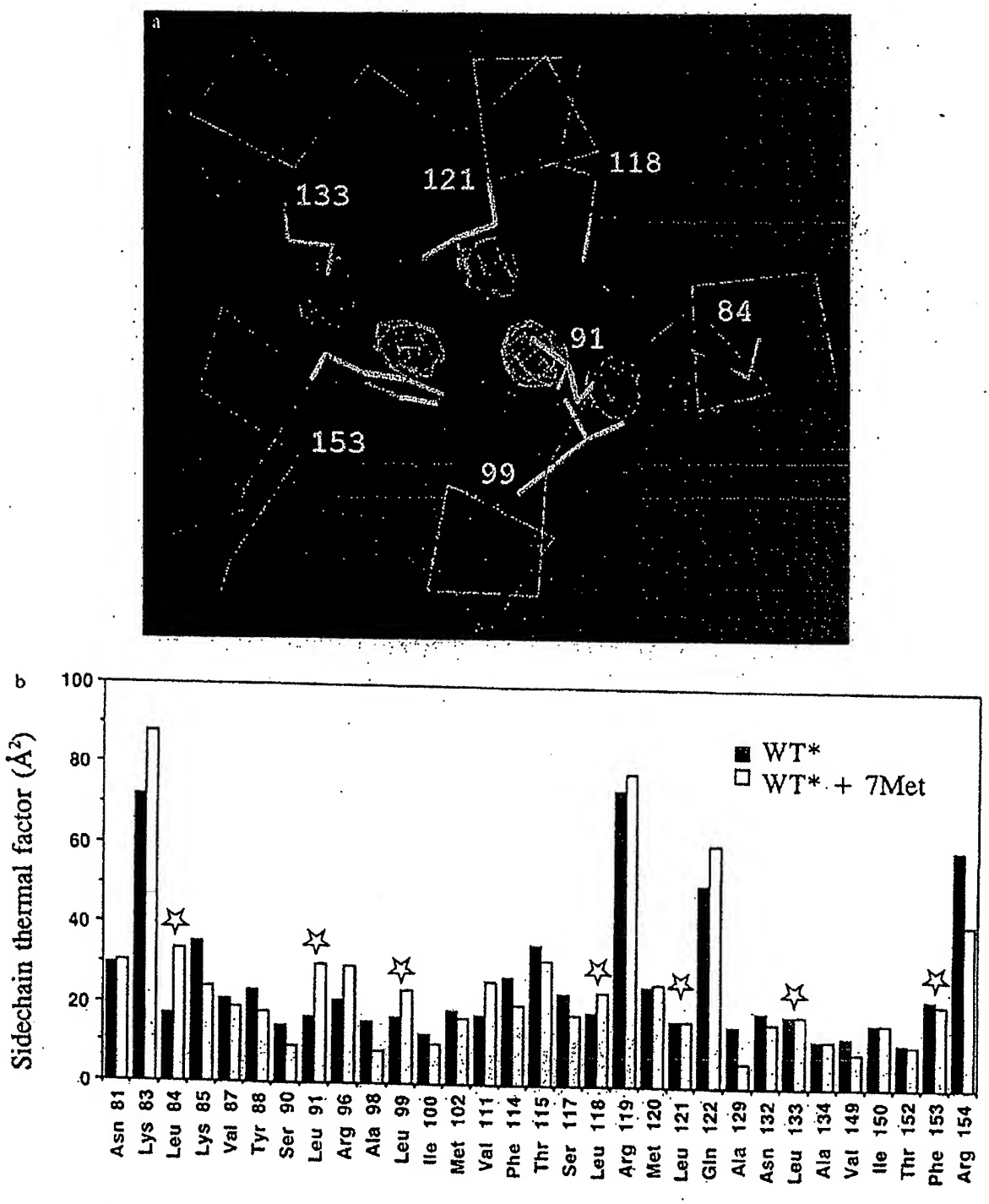

FIG. 2. (*a*) Map showing the difference in electron density between the seven-methionine mutant and wild-type T4 lysozyme. Coefficients ($F_{mut} - F_{WT*}$) where F_{mut} and F_{WT*} correspond to the observed structure amplitudes of the mutant and wild-type structures. Phases from the refined structure of WT* lysozyme (18). Resolution is 1.9 Å. Blue contours representing positive density are drawn at $+3\sigma$ where σ is the root-mean-square density throughout the unit cell. Red contours (negative density) are drawn at -3σ. Superimposed is the structure of the carboxyl-terminal domain of WT* lysozyme with the backbone shown in green and the substituted side chains in yellow. Crystals were obtained using ~2M phosphate solutions, ~pH 6.7 (18). (*b*) Comparison of the thermal factors of side chains within the core of the 7-methionine mutant (open bars) with those of wild-type lysozyme (solid bars). The figure includes the seven residues that were changed to methionine (marked with stars) as well as all residues within 4 Å of the substituted amino acids. The amino acids are identified as in the WT* structure.

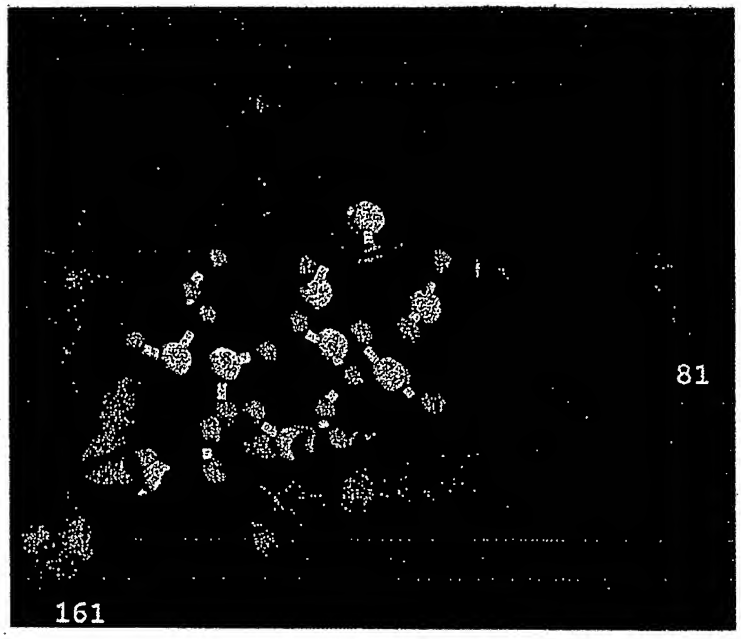

FIG. 3. Residues 81–161 of the carboxyl-terminal domain of T4 lysozyme. The figure shows the distribution of methionines within the hydrophobic core of the molecule. It illustrates the structure of the mutant in which seven methionines have been introduced genetically and includes two additional methionines that are present in the native protein. The methionine side chains are shown in green with the sulfur atoms in yellow. The carboxyl-terminal domain of T4 lysozyme contains a single, completely buried, methionine (Met-102), and two more (Met-106 and -120) that are about 80% buried. In addition, six leucines (Leu-84, -91, -99, -118, -121, and -133), two isoleucines (Ile-78 and -100), one phenylalanine (Phe-153), and one valine (Val-103) were chosen for substitution with methionine.

single type of amino acid are possible in the core as well, albeit with a progressive loss of stability. It suggests that it may be possible to replace the overall amino acid sequence of a protein with a much simpler sequence based on a subset of the 20 naturally occurring amino acids. Perhaps this may be a way to simplify the protein folding problem.

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1. Richards, F. M. (1974) *J. Mol. Biol.* **82**, 1–14.
2. Ponder, J. W. & Richards, F. M. (1987) *J. Mol. Biol.* **193**, 775–791.
3. Behe, M. J., Lattman, E. E. & Rose, G. D. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4195–4199.
4. Crick, F. H. C. (1953) *Acta Crystallogr.* **6**, 689–697.
5. Chothia, C., Levitt, M. & Richardson, D. (1981) *J. Mol. Biol.* **145**, 215–250.
6. Harbury, P. B., Zhang, T., Kim, P. S. & Alber, T. (1993) *Science* **262**, 1401–1407.
7. Harrison, S. C. & Durbin, R. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4028–4030.
8. Kellis, J. T., Nyberg, K., Sali, D. & Fersht, A. R. (1988) *Nature (London)* **333**, 784–786.
9. Shortle, D., Stites, W. E. & Meeker, A. K. (1990) *Biochemistry* **29**, 8033–8041.
10. Eriksson, A. E., Baase, W. A., Zhang, X-J., Heinz, D. W., Blaber, M., Baldwin, E. P. & Matthews, B. W. (1992) *Science* **255**, 178–183.
11. Lim, W. A. & Sauer, R. T. (1991) *J. Mol. Biol.* **219**, 359–376.
12. Baldwin, E. P., Hajiseyedjavadi, O., Baase, W. A. & Matthews, B. W. (1993) *Science* **262**, 1715–1718.
13. Pielak, G. J., Auld, D. S., Beasley, J. R., Betz, S. F., Cohen, D. S., Doyle, D. F., Finger, S. A., Fredericks, Z. L., Hilgen-Willis, S., Saunders, A. J. & Trojak, S. K. (1995) *Biochemistry* **34**, 3268–3276.
14. Axe, D. D., Foster, N. W. & Fersht, A. R. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 5590–5594.
15. Klapper, M. H. (1977) *Biochem. Biophys. Res. Commun.* **78**, 1018–1024.
16. Kuwajima, K. (1989) *Proteins* **6**, 87–103.
17. Poteete, A. R., Dao-pin, S., Nicholson, H. & Matthews, B. W. (1991) *Biochemistry* **30**, 1425–1432.
18. Eriksson, A. E., Baase, W. A. & Matthews, B. W. (1993) *J. Mol. Biol.* **229**, 747–769.
19. Hamlin, R. (1985) *Methods Enzymol.* **114**, 416–452.
20. Zhang, X-J. & Matthews B. W. (1993) *J. Appl. Cryst.* **26**, 457–462.
21. Tronrud, D. E., Ten Eyck, L. F. & Matthews, B. W. (1987) *Acta Crystallogr. A* **43**, 489–503.
22. Tronrud, D. E. (1996) *J. Appl. Cryst.* **29**, 100–104.
23. Fauchère, J-L. & Pliška, V. (1983) *Eur. J. Med. Chem.* **18**, 369–375.
24. Creamer, T. P. & Rose, G. D. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5937–5941.
25. Sternberg, M. J. E. & Chickos, J. S. (1994) *Protein Eng.* **7**, 149–155.
26. Hendrickson, W. A. (1991) *Science* **254**, 51–58.
27. Blaber, M., Baase, W. A., Gassner, N. & Matthews, B. W. (1995) *J. Mol. Biol.* **246**, 317–330.
28. Zhang, X-J., Baase, W. A., Shoichet, B. K., Wilson, K. P. & Matthews, B. W. (1995) *Protein Eng.* **8**, 1017–1022.
29. Streisinger, G., Mukai, F., Dreyer, W. J., Miller, B. & Horiuchi, S. (1961) *Cold Spring Harbor Symp. Quant. Biol.* **26**, 25–30.

Appendix B

Biochemistry

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Perspectives in Biochemistry

Additivity of Mutational Effects in Proteins

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The energetics of virtually all binding functions in proteins is the culmination of a set of molecular interactions. For example, removal of a single molecular contact by a point mutation causes relatively small reductions (typically 0.5–5 kcal/mol) in the free energy of transition-state stabilization [for reviews see Fersht (1987) and Wells and Estell (1988)], protein–protein interactions (Laskowski et al., 1983, 1989; Ackers & Smith, 1985), or protein stability [for review see Matthews (1987)] compared to the overall free energy associated with these functional properties (usually 5–20 kcal/mol). Thus, it is possible to modulate protein function by mutation at many contact sites. In fact, to design large changes in function will often require mutation of more than one functional residue.

There is now a large data base for free energy changes that result when single mutants are combined. A review of these data shows that, in the majority of cases, the sum of the free energy changes derived from the single mutations is nearly equal to the free energy change measured in the multiple mutant. However, there are two major exceptions where such simple additivity breaks down. The first is where the mutated residues interact with each other, by direct contact or indirectly through electrostatic interactions or structural perturbations, so that they no longer behave independently. The second is where the mutation causes a change in mechanism or rate-limiting step of the reaction. It is important to note that the additive effects discussed here do not change the molecularity of their respective reactions. When the molecularity of the reaction changes [as in comparing the free energy of binding of one linked substrate (A–B) versus the sum of two fragments (A plus B)], large deviations from simple additivity can result from entropic effects (Jencks, 1981). Although the focus here is on enzyme activity, similar conclusions may be drawn from mutations affecting protein–protein interactions, protein–DNA recognition, or protein stability. Some practical examples and applications are discussed.

Additivity Relationships

The change in free energy of a functional property caused by a mutation at site X is typically expressed relative to that of the wild-type protein as $\Delta\Delta G_{(X)}$. Such free energy changes for two single mutants (X and Y) can be related to those of a double mutant (designated X,Y) by eq 1 (Carter et al., 1984; Ackers & Smith, 1985). The ΔG_I term (also called the coupling energy; Carter et al., 1984) should reflect the extent to which the change in energy of interaction between sites X and Y affects the functional property measured. It is possible for ΔG_I to be either positive or negative depending upon whether the interactions between the mutant side chains reduce or enhance the functional property measured. Furthermore, the ΔG_I term should not exceed the free energy of interaction between side chains at sites X and Y except in cases where these mutations cause large structural perturbations. This was first applied to evaluating the functional independence of residues mutated in tyrosyl-tRNA synthetase (Carter et al., 1984). In one case the sum of the $\Delta\Delta G$ values for single mutants was equal to that of the double mutant, indicating the sites functioned independently; in another example there was a large discrepancy, suggesting the sites were interacting.

$$\Delta\Delta G_{(X,Y)} = \Delta\Delta G_{(X)} + \Delta\Delta G_{(Y)} + \Delta G_I \quad (1)$$

Simple Additivity in Transition-State Binding Interactions

The strengths of noncovalent interactions are strongly dependent upon the nature of the two groups and the distance (r) between them. For example, the free energy of charge–charge, random charge–dipole, random dipole–dipole, van der Waals attraction, and repulsion decay as $1/r$, $1/r^4$, $1/r^6$, $1/r^6$, and $1/r^{12}$, respectively [for review see Fersht (1985)]. Thus, when the side chains at sites X and Y are remote to one another and assuming no large structural perturbations, the ΔG_I term should be negligible and eq 1 thus simplifies to

$$\Delta\Delta G_{(X,Y)} \cong \Delta\Delta G_{(X)} + \Delta\Delta G_{(Y)} \quad (2)$$

This situation, here referred to as simple additivity, is generally observed except where side chains are close to each other or when one or both of the mutants change the rate-limiting step or reaction mechanism. These principles are well illustrated from data of additive mutational effects on transition-state stabilization energies.

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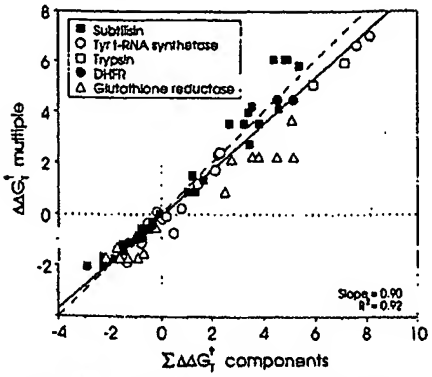

FIGURE 1: Plot of the changes in transition-state stabilization energies for the multiple mutant versus the sum for the component mutants. Data are taken from Table I and represent mutants from subtilisin (■), tyrosyl-tRNA synthetase (○), trypsin (□), DHFR (●), and glutathione reductase (△), where mutant or wild-type side chains should not contact one another. The dashed line has a slope of 1, and the solid line is a best fit to all the data.

Changes in transition-state stabilization energy ($\Delta\Delta G_T^\ddagger$) caused by a mutation can be calculated from eq 3 (Wilkinson et al., 1983), in which R is the gas constant, T is the absolute

$$\Delta\Delta G_T^\ddagger = -RT \ln \frac{(k_{cat}/K_M)_{mutant}}{(k_{cat}/K_M)_{wild\text{-}type}} \quad (3)$$

temperature, k_{cat} is the turnover number, and K_M is the Michaelis constant for the mutant and wild-type enzyme against a fixed substrate. $\Delta\Delta G_T^\ddagger$ represents the change in free energy to reach the transition-state complex ($E{\cdot}S^\ddagger$) from the free enzyme and substrate (E + S).

To analyze the proposition that the interaction energy term, $\Delta G_{T(I)}^\ddagger$, is relatively small when the sites of mutation (X and Y) are remote to one another, $\Delta\Delta G_T^\ddagger$ values were collected from the literature where side-chain substitutions in the multiple mutant are beyond van der Waals contact (>4 Å distant) from each other (Table I). There are at least 25 examples distributed across five different enzymes where $\Delta\Delta G_T^\ddagger$ values can be calculated for the individual and multiple mutants assayed in at least two different ways. Among these are examples where electrostatic interactions, hydrogen bonding, and steric and hydrophobic effects have been altered separately or in combination with others. The X-ray structures of the wild-type proteins show that the wild-type side chains are not in contact. Modeling suggests the mutant side chains are beyond possible van der Waals contact unless the mutant side chains were to cause significant changes in the overall protein structure. Such large changes are rarely observed in structures of site-specific mutant proteins (Katz & Kossiakoff, 1986; Alber et al., 1987; Howell et al., 1986; Wilde et al., 1988) or even highly variant natural proteins (Chothia & Lesk, 1986).

A collective plot of the sum of the $\Delta\Delta G_T^\ddagger$ values for the component mutants versus the corresponding multiple mutant (Table I) gives a remarkably strong correlation ($R^2 = 0.92$) with a slope near unity (Figure 1). The simplest interpretation is that the interaction term, $\Delta G_{T(I)}^\ddagger$, is small compared to the overall effects on $\Delta\Delta G_{T(X,Y)}^\ddagger$. It is formally possible that there are large and compensating effects between side chains X and Y that systematically lead to small net values for $\Delta G_{T(I)}^\ddagger$.

There are some notable exceptions that weaken the correlation within the data set (Table I). In particular, combining the R204L mutation in *Escherichia coli* glutathione reductase gives a less than additive effect, especially when combined with another mutant, R198M (Scrutton et al., 1990). These basic residues are not in direct contact, but both side chains form a salt bridge with the 2′-phosphate group of NADPH. Indeed, the largest discrepancies are when these mutants are assayed with NADPH as compared to NADH. Similarly, the sum of the $\Delta\Delta G_T^\ddagger$ values for two positively charged component mutants in subtilisin (D99K and E156K) overestimates the effect of the multiple mutant when assayed with an Arg but not with a Phe substrate (Russell & Fersht, 1987). Such discrepancies are not too surprising because charge–charge interactions fall off as $1/r$ and can exhibit long-range effects in proteins [for example, see Russell and Fersht (1988)]. The physical basis for other large discrepancies not involving electrostatic substitutions is less clear but may involve unexpectedly large structural changes or changes in enzyme mechanism (see below).

These additivity tests are not particularly dominated by one of the single mutants in the sum. The average contribution (±SE) for the most dominant mutant in ech sum calculated from the 69 additivity tests given in Table I is only 68% (±15%) of the total sum (theoretical is ~50%). Furthermore, the plot in Figure 1 is not analogous to graphs of correlated variables, where A is plotted versus the sum of A + B, because in Figure 1 the values on the y-axis are determined *independently* from those on the x-axis.

Complex Additivity in Transition-State Stabilization—When $\Delta G_{T(I)}^\ddagger \neq 0$

(A) Change in Interaction Energy between Sites X and Y. Where residues X and Y are close enough to contact, it is more likely that the $\Delta G_{T(I)}^\ddagger$ term will be significant. There are 11 examples collectively from tyrosyl-tRNA synthetase and subtilisin that fit this category (Table II).

A series of mutants in tyrosyl-tRNA synthetase at positions 48 and 51 (Carter et al., 1984; Lowe et al., 1985) show complex additivity (Table II). His48 and Thr51 in the wild-type structure are next to each other on adjacent turns of an α-helix. His48 hydrogen bonds to the ribose ring oxygen of ATP while Thr51 can make van der Waals contact with ATP. The T51P mutation increases the catalytic efficiency of the enzyme in some assays by more than −2 kcal/mol (Wilkinson et al., 1984). However, when this mutation is combined with mutations at position 48, the effects are not simply additive. An X-ray structure of the T51P mutant indicates there are no structural changes in the α-helix (Brown et al., 1987). Instead, it is suggested that the T51P mutant is improved over wild type because the wild-type enzyme contains a bound water in the vicinity of Thr51 that disfavors substrate binding. Blow and co-workers (Brown et al., 1987) argue that the change in solvent structure propagated to position 48 may account for the complex additivity. In the previous section, the double mutant (H48G,T51A) exhibited nearly simple additivity (Table I). Presumably, the smaller and less hydrophobic alanine substitution at position 51 should not introduce as large a change in solvent structure as the pyrrolidone ring of proline.

In the case of subtilisin (Table II), Glu156 is near the top of the P1 binding crevice while Gly166 is at the bottom. In the wild-type enzyme these sites do not make direct van der Waals contact, but large side chains substituted at position 166 can be modeled to contact the residue at position 156. In fact, X-ray structural analysis shows that an Asn side chain at position 166 makes a good hydrogen bond with Glu156 (Bott et al., 1987). Moreover, all of the substitutions are polar or charged, the energetics of which are expected to be the most long range. Thus, the mutant side chains alter substantially the intramolecular interactions between positions 156 and 166.

Table I: Comparison of Sums of $\Delta\Delta G_T^{\ddagger}$ from Component Mutants vs the Multiple Mutant Where the Mutant or Wild-Type Side Chains *Do Not* Contact One Another

assay	$\Delta\Delta G_T^{\ddagger}$ component mutants			sum	multiple mutant
Tyrosyl-tRNA Synthetase					
	C35G + H48G[a]				
ATP/PP_i	+1.20	+1.04		+2.24	+2.30
ATP/tRNA	+1.05	+1.13		+2.18	+1.68
Tyr/PP_i	+1.14	+1.12		+2.26	+2.32
Tyr/tRNA	+0.32	+1.12		+1.45	+1.20
	C35G + T51P				
ATP/PP_i	+1.20	−1.91		−0.71	−1.14
ATP/tRNA	+1.05	−2.35		−1.30	−1.88
Tyr/PP_i	+1.14	−0.64		+0.50	−0.74
Tyr/tRNA	+0.32	+0.50		+0.82	+0.21
	C35G + T51C[b]				
ATP/tRNA	+1.05	−0.93		+0.12	−0.22
ATP/Tyr	+1.14	−0.91		+0.23	−0.13
	H48N + T51A[c]				
ATP/PP_i	+0.26	−0.38		−0.12	+0.04
ATP/tRNA	−0.13	−0.32		−0.45	−0.37
	T40A + H45G[d]				
Tyr/Tyr	+5.02	+3.15		+8.17	+6.95
ATP/Tyr	+5.13	+2.44		+7.57	+6.67
Rat Trypsin					
	G216A + G226A[e]				
K	+2.75	+3.13		+5.88	+5.07
R	+2.19	+4.91		+7.10	+5.90
Dihydrofolate Reductase ($\Delta\Delta G_{binding}$)					
	F31V + L54G[f]				
H_2F	+1.6	+2.9		+4.5	+4.5
MTX	+2.2	+2.9		+5.1	+4.5
Subtilisin BPN′					
	E156S + Y217L + G169A[g]				
E	−1.43	−0.87	−0.62	−2.92	−2.06
Q	−0.60	−0.36	−0.32	−1.28	−1.14
A	−0.15	−0.41	−0.27	−0.83	−0.92
K	+1.70	−0.08	−0.30	+1.32	+0.87
M	−0.86	−0.32	−0.39	−1.57	−1.41
F	−0.61	−0.29	−0.66	−1.56	−1.17
Y	−0.24	−0.12	−0.41	−0.77	−0.59
	E156S + Y217L				
E	−1.43	−0.87		−2.30	−1.67
Q	−0.60	−0.36		−0.96	−0.96
A	−0.15	−0.41		−0.56	−0.53
K	+1.70	−0.08		+1.62	+1.33
M	−0.86	−0.32		−1.18	−1.11
F	−0.61	−0.29		−0.90	−0.84
Y	−0.24	−0.12		−0.36	−0.32
	E156S, Y217L + G169A				
E	−1.67	−0.62		−2.29	−2.06
Q	−0.96	−0.32		−1.28	−1.14
A	−0.53	−0.27		−0.80	−0.92
K	+1.33	−0.30		+1.03	+0.87
M	−1.11	−0.39		−1.50	−1.41
F	−0.84	−0.66		−1.50	−1.17
Y	−0.32	−0.41		−0.73	−0.59
	D99S + E156S[h]				
R	+0.47	+0.77		+1.24	+1.52
F	0	−0.62		−0.62	−0.52
Subtilisin BPN′					
	D99K + E156K				
R	+1.29	+2.12		+3.41	+2.74
F	+0.13	−0.49		−0.36	−0.42
	G166A + E156S, G169A, Y217L[i]				
F	−0.40	−1.46		−1.86	−1.76
Y	+0.94	−1.03		−0.09	+0.02
	G166A + S24C, H64A				
F	−0.40	+4.96		+4.56	+4.11
Y	+0.94	+4.40		+5.34	+5.84
	E156S, G169A, Y217L + S24C, H64A				
F	−1.46	+4.96		+3.50	+4.21
Y	−1.03	+4.40		+3.37	+3.96
	S24C, H64A, G169A, Y217L + G166A				
F	+4.21	−0.40		+3.81	+3.53
Y	+3.96	+0.94		+4.90	+6.07
	S24C, H64A, + E156S, G169A, G166A Y217L				
F	+4.11	−1.46		+2.65	+3.53
Y	+5.84	−1.03		+4.81	+6.07
	S24C, H64A + E156S, G166A, G169A, Y217L				
F	+4.96	−1.76		+3.20	+3.53
Y	+4.40	+0.02		+4.38	+6.07
***E. coli* Glutathione Reductase**					
	A179G + R198M[j]				
NADH	−1.10	−0.62		−1.72	−1.32
NADPH	+0.08	+2.68		+2.76	+2.11
	A179G + R204L				
NADH	−1.10	+0.41		−0.69	−1.54
NADPH	+0.08	+2.42		+2.50	+0.87
	R198M + R204L				
NADH	−0.62	+0.41		−0.21	−0.51
NADPH	+2.68	+2.42		+5.10	+3.70
	A179G + R179M, R204L				
NADH	−1.10	−0.51		−1.61	−1.72
NADPH	+0.08	+3.70		+3.78	+2.22
	R198M + A179G, R204L				
NADH	−0.62	−1.54		−2.16	−1.72
NADPH	+2.68	+0.87		+3.55	+2.22
	R204L + A179G, R198M				
NADH	+0.41	−1.32		−0.91	−1.72
NADPH	+2.42	+2.11		+4.53	+2.22
	R179G + R198M + R204L				
NADH	−1.10	−0.62	+0.41	−1.31	−1.72
NADPH	+0.08	+2.68	+2.42	+5.18	+2.22

[a] Carter et al. (1984). The assays refer to measurements of ATP-dependent pyrophosphate exchange (ATP/PP_i) or tRNA charging (ATP/tRNA) under saturating conditions for tyrosine and vice versa for Tyr/PP_i exchange and Tyr/tRNA charging. [b] Lowe et al. (1985). The ATP/Tyr activation assay refers to formation of tyrosyl adenylate under saturating concentrations of tyrosine. [c] Jones et al. (1986). [d] Leatherbarrow et al. (1986). The ATP/Tyr and Tyr/Tyr activation assays refer to formation of tyrosyl adenylate under pre-steady-state conditions, and k_{cat}/K_M is calculated from k_3/K_S for tyrosine and ATP, respectively. [e] Craik et al. (1985). The substrate was D-Val-Leu-(X)-aminofluorocoumarin where the P1 residue (X) is either Lys (K) or Arg (R). [f] Mayer et al. (1986). The ligand was either dihydrofolate (H_2F) or methotrexate (MTX). [g] Wells et al. (1987a). The substrate was succinyl-L-Ala-L-Ala-L-Pro-L-(X)-*p*-nitroanilide where the P1 (X) residue (Schechter & Berger, 1937) was either Glu (E), Gln (Q), Ala (A), Lys (K), Met (M), Phe (F), or Tyr (Y). [h] Russell and Fersht (1987). The substrate was benzoyl-L-Val-Gly-L-Arg-*p*-nitroanilide (R) or succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (F). [i] Carter et al. (1989). The substrate was succinyl-L-Phe-L-Ala-L-His-L-(X)-*p*-nitroanilide where X was either Phe (F) or Tyr (Y). [j] Scrutton et al. (1990). The assay followed the reduction of oxidized glutathione by NADH or NADPH.

Table II: Comparison of Sums of $\Delta\Delta G_T^{\ddagger}$ from Component Mutants vs the Multiple Mutant Where the Mutant Side Chains *Can* Contact One Another

assay[a]	$\Delta\Delta G_T^{\ddagger}$ component mutants		sum	multiple mutant
	Tyrosyl-tRNA Synthetase			
	H48G + T51P[b]			
ATP/PP_i	+1.04	−1.91	−0.87	+1.07
ATP/tRNA	+1.13	−2.35	−1.22	+0.77
Tyr/PP_i	+1.12	−0.64	+0.48	+1.02
Tyr/tRNA	+1.12	+0.50	+1.63	+0.17
ATP/Tyr[c]	+0.95	−1.99	−1.04	+1.04
Tyr/ATP	+1.07	−0.38	+0.69	+0.82
	H48N + T51P			
ATP/Tyr	+0.18	−1.99	−1.81	−0.76
Tyr/Tyr	+0.36	−0.38	−0.02	−0.64
ATP/tRNA	−0.02	−2.23	−2.25	−1.07
	N48G + T51P			
ATP/Tyr	+0.37	−0.94	−0.57	+0.86
Tyr/Tyr	+0.41	−1.00	−0.59	+0.45
ATP/tRNA	+1.26	−1.05	+0.21	+0.90
	Q48G + T51P			
ATP/Tyr	−1.31	−1.09	−2.40	−1.22
Tyr/Tyr	−2.05	−1.65	−3.70	−2.31
ATP/tRNA	−1.87	−1.85	−3.72	−2.23
	H48Q + T51P			
ATP/Tyr	+2.26	−1.99	+0.27	+1.17
Tyr/Tyr	+3.13	−0.38	+2.75	+1.48
ATP/tRNA	+3.11	−2.23	+0.88	+1.26
	Subtilisin BPN'			
	E156Q + G166D[d]			
Q	−1.04	+1.27	+0.23	+0.75
M	−0.45	+1.83	+1.38	+0.16
K	+2.15	+0.53	+2.68	+0.26
	E156S + G166D			
Q	−0.59	+1.27	+0.68	+0.74
M	−0.85	+1.83	+0.98	+0.66
K	+1.68	+0.53	+2.22	+0.49
	E156Q + G166N			
E	−1.71	−0.11	−1.82	−0.69
Q	−1.04	+0.14	−0.90	−0.77
M	−0.45	+0.18	−0.27	−1.10
K	+2.15	+0.48	+2.73	+1.16
	E156S + G166N			
E	−1.44	−0.11	−1.55	−0.51
Q	−0.59	+0.14	−0.45	−0.85
M	−0.85	+0.18	−0.67	−0.78
K	+1.68	+0.48	+2.16	+1.26
	E156S + G166K			
E	−1.44	−3.49	−4.93	−4.49
Q	−0.59	−1.03	−1.62	−0.95
M	−0.85	−1.37	−2.22	−1.12
K	+1.68	+0.51	+2.19	+1.88
	E156Q + G166K			
E	−1.71	−3.49	−5.20	−4.49
Q	−1.04	−1.03	−2.07	−0.95
M	−0.45	−1.37	−1.82	−1.12
K	+2.15	+0.51	+2.66	+1.88

[a] See Table I for description assays. [b] Lowe et al. (1985). [c] Carter et al. (1984). [d] Wells et al. (1987b).

In these six examples there are large and systematic discrepancies between the sum of the $\Delta\Delta G_T^{\ddagger}$ values for the single mutants and those of the corresponding double mutant (Wells et al., 1987b). In almost all cases, the sum of the $\Delta\Delta G_T^{\ddagger}$ values for the single mutants is much greater than the value for the multiple mutant. Nonetheless, the $\Delta\Delta G_T^{\ddagger}$ value predicted from the sum of the single mutants does have the same sign as that for the double mutant, so that the single mutants predict qualitatively the effect on the multiple mutant.

A plot (Figure 2) of the collective data set from Table II is in contrast to that seen in Figure 1. The $\Delta\Delta G_T^{\ddagger}$ values for the multiple mutants correlate more poorly with the sum of

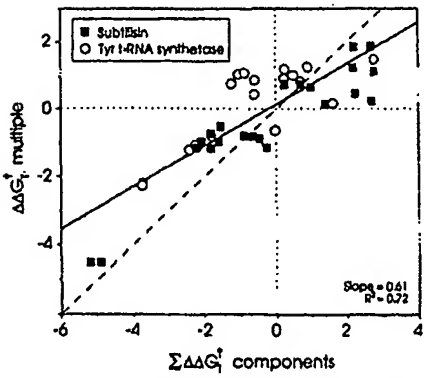

FIGURE 2: Data are taken from Table II for mutants of subtilisin (■) or tyrosyl-tRNA synthetase (○) where mutant or wild-type side chains can contact each other. The dashed line represents a theoretical line of unity slope, and the solid line represents the best fit.

the component single mutants ($R^2 = 0.72$). Moreover, the slope of the line (0.61) is much below unity. This indicates that the function of one residue is compromised by mutation of another. Of the 40 additivity examples, the average contribution of the most dominant single mutant to the sum of the $\Delta\Delta G_T^{\ddagger}$ values is 71% (±13%) of the total. Thus (as in Figure 1), both single mutants can contribute substantially to free energy changes measured in the multiple mutant. However, this data set is derived from mutations at only two different sites on two different proteins.

In summary, complex additivity can be observed when mutations at sites X and Y change the intramolecular interaction energy between sites. This can be mediated by direct steric, electrostatic, hydrogen-bonding, or hydrophobic interactions or indirectly through large structural changes in the protein, solvent shell, or electrostatic interactions. Complex additivity is most likely to occur where the sites of mutation are very close together and larger or chemically divergent side chains are introduced.

(B) Mutations at Sites X or Y Change the Enzyme Mechanism or Rate-Limiting Step. If the catalytic functions of two or more residues are interdependent, then a mutation of one residue can affect the functioning of the other(s). This form of complex additivity is well illustrated for mutations in the catalytic triad and oxyanion binding site of subtilisin (Carter & Wells, 1988, 1990). In the catalytic mechanism of subtilisin (Figure 3), the rate-limiting step in amide bond hydrolysis is transfer of the proton from Ser221 to His64 with nucleophilic attack upon the scissile carbonyl carbon. This is accompanied by electrostatic stabilization of the protonated imidazole by Asp32 and hydrogen bonding to the oxyanion by the side chain of Asn155 and the main-chain amide of Ser221. Mutational analysis shows that once the catalytic Ser221 is mutated to Ala (S221A), additional mutations in the triad or oxyanion binding site cause no further loss in catalytic efficiency (Table III).

The S221A enzyme retains a catalytic activity that is still 10^4 above the solution hydrolysis rate (Carter & Wells, 1988). It is proposed that this residual activity is derived from remaining transition-state binding contacts outside of the catalytic triad coupled with solvent attack upon the carbonyl carbon from the face opposite position 221 (Carter & Wells, 1990). This proposal is based on a model showing that there is no room for a water molecule near Ala221 once the substrate is bound. Furthermore, conversion of Asn155 to Gly enhances the activity of the S221A mutant by −1.2 kcal/mol (Table III).

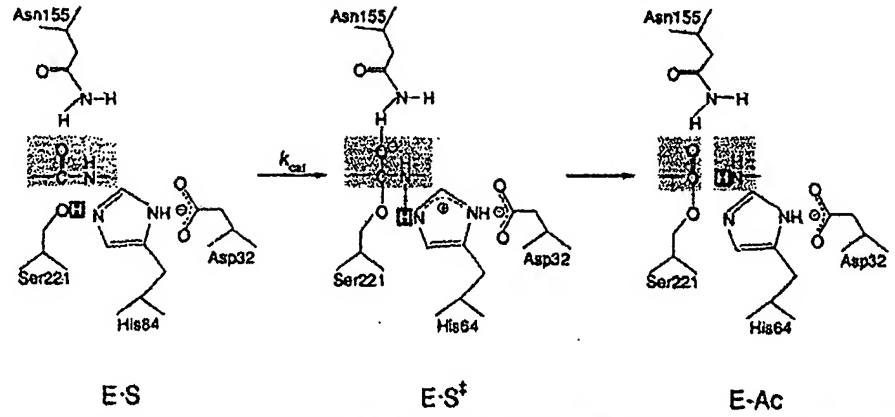

FIGURE 3: Schematic diagram of the mechanism of subtilisin showing the rate-limiting acylation step for hydrolysis of peptide bonds. Reproduced with permission from Carter and Wells (1988). Copyright 1988 Macmillan.

Table III: Comparison of Sums of $\Delta\Delta G_T^{\ddagger}$ from Component Mutants vs the $\Delta\Delta G_T^{\ddagger}$ for Multiple Mutants in the Catalytic Triad and Oxyanion Binding Site of Subtilisin BPN'[a]

component mutants	sum	multiple mutant
S221A + H64A[b]		
+8.93 +8.84	+17.76	+8.83
S221A + D32A		
+8.93 +6.52	+15.45	+8.86
H64A + D32A		
+8.84 +6.52	+15.36	+7.48
S221A + H64A + D32A		
+8.93 +8.84 +6.52	+24.29	+8.65
S221A + H64A, D32A		
+8.93 +7.48	+16.40	+8.65
H64A + S221A, D32A		
+8.84 +8.86	+17.70	+8.65
D32A + S221A, H64A		
+6.52 +8.83	+15.35	+8.65
S221A + N155G[c]		
+8.93 +3.08	+12.01	+7.70

[a] All enzymes were assayed with the substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide. [b] Carter and Wells (1988). [c] Carter and Wells (1990).

This is consistent with the opposite-face solvent attack mechanism of S221A, because the oxyanion (Figure 3) would develop away from Asn155 and the N155G mutation improves solvent accessibility to the scissile carbonyl carbon.

Complex additivity is also seen for subtilisin mutated at positions 64 and 32. The double (H64A,D32A) and corresponding single mutants show a linear dependence upon hydroxide ion concentration (between pH 8 and 10) that may reflect hydroxide assistance in the deprotonation of the Oγ of Ser221 (Carter & Wells, 1988). Thus, once His64 is converted to Ala, Asp32 is a liability, presumably by electrostatic repulsion of hydroxide ion. [Note the −1.3 kcal/mol improvement in $\Delta\Delta G_T^{\ddagger}$ for the double mutant (H64A,D32A) compared to H64A alone; Table III.]

In summary, if an enzyme mechanism relies upon cooperative interaction between two or more residues, then multiple mutations within this subset can result in large values for $\Delta G_{T(I)}^{\ddagger}$. In fact, if the mechanism is changed substantially, residues that were a catalytic asset can become a liability. Simple additivity can also break down when one or more of the mutations cause a change in the rate-limiting step. In an extreme case, one may have a number of mutants in an enzyme that enhance the activity, but the cumulative enhancement of activity could not go beyond the diffusion-controlled limit (Albery & Knowles, 1976).

Additive Effects on Substrate Binding

The analysis above considered changes in binding free energies between the free enzyme and substrate (E + S) to yield the bound transition-state complex (E-S‡). The steady-state kinetic analysis for subtilisin and tyrosyl-tRNA synthetase is such that the K_M values approximate the enzyme–substrate dissociation constant K_s. Additivity analysis based on calculations of $\Delta\Delta G_{binding}$ (from K_M values) or $\Delta\Delta G_{cat}$ (from k_{cat} values) yields qualitatively the same results (not shown) as shown in Tables I and II and Figures 1 and 2. Thus, deviations from simple additivity are not systematically found in either the energetics to form the E-S complex or those to reach E-S‡.

Additive Effects on Protein–Protein Interactions

The first clear examples of additive binding effects caused by amino acid replacements in proteins were reported by Laskowski et al. (1983) and reviewed by others (Ackers & Smith, 1985; Horovitz & Rigbi, 1985). One hundred natural variants of a proteinase inhibitor, the ovomucoid third domain, have been isolated and sequenced from the eggs of different bird species (Empie & Laskowski, 1982; Laskowski et al., 1987). This is a nested set of proteins because for any one of these avian inhibitors there is a close relative containing only one or a few amino acid substitutions. Moreover, the association constants (K_a) of these inhibitors with a variety of serine proteinases vary over an enormous range (10^9-fold). Laskowski et al. (1983, 1989) have shown that the effect of a given residue replacement on K_a is about the same irrespective of the inhibitor scaffold the replacement is made in.

In addition to ovomucoid, four additivity examples have been constructed from natural variants at the subunit interface of tetrameric hemoglobin (Ackers & Smith, 1985). Three additivity examples have been analyzed for interactions of hGH with its receptor (B. C. Cunningham and J. A. Wells, unpublished results) and one example for association of synthetic variants of the RNase S peptide with RNase S protein (Mitchinson & Baldwin, 1986). The entirety of this data set is not tabulated because much on the ovomucoid inhibitors and hGH is unpublished. Nonetheless, these researchers were kind enough to provide their data formatted so it could be plotted collectively in Figure 4. These data consist of 91 additivity examples (80 in ovomucoids alone), representing 22 multiple mutants across four different proteins, and span a wide range of change in binding free energy (−10 to +7

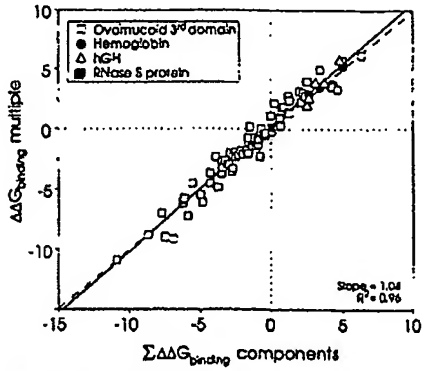

FIGURE 4: Plot showing the sum of changes in free energies of binding at protein–protein interfaces for component mutants versus the corresponding multiple mutant. Data represent interactions between ovomucoid third domain and various serine proteases (□) (R. Wynn and M. Laskowski, personal communication), regulatory interface of $\alpha_2\beta_2$ hemoglobin (●) (Ackers & Smith, 1985), hGH and its receptor (stippled △) (B. Cunningham and J. Wells, personal communication), and RNase S peptide and S protein (■) (Mitchinson & Baldwin, 1986). The dashed line represents a line of unity slope, and the solid line is the best fit.

kcal/mol). The plot shows a very strong linear correlation (R^2 = 0.96) with a slope near unity. Although the data for the ovomucoid were not sorted to evaluate changes at intramolecular contact sites, most are not expected to be in contact, and all of the other examples represent noncontact sites. Thus, the large data base derived from natural variants of ovomucoid third domain, as well as a smaller number of examples from several other proteins, indicates that multiple mutations at protein–protein interfaces commonly produce simple additive effects.

Additive Effects in DNA–Protein Interactions

One of the clear advantages in analyzing DNA–protein interactions is the ability to apply powerful selections that make analysis by random mutational studies feasible. Additivity in DNA–protein interactions was first demonstrated by reversion analysis of λ repressor (Nelson & Sauer, 1985). A mutation that decreased the binding affinity for the λ operator site (K4Q) was reverted by mutations at several second sites (E34K, G48S, and E83K). When these second-site revertants were introduced into wild-type λ repressor, they caused increases in affinity similar to those observed in the first-site suppressor mutant (K4Q).

Functional independence for mutations at DNA–protein contacts has been demonstrated by additive effects for mutants of CAP (catabolite gene activator protein) and its operator sequence (Ebright et al., 1987) as well as *lac* repressor and its corresponding operator sequence (Ebright, 1986). Simple additivity of mutational effects in the operator sequences for Cro repressor (Takeda et al., 1989) and λ repressor (Sarai & Takeda, 1989) has been most systematically demonstrated. Simple additivity has also been reported for multiple mutations in the *lac* repressor (Lehming et al., 1990). In fact, simple additivity is so predictable in DNA–protein interactions that the observation of complex additivity has been used to predict specific DNA–protein contacts in the *lac* repressor–operator complex (Ebright, 1986).

Additive Effects on Protein Stability

The first systematic analysis of additive effects of site-specific mutations on protein stability was reported by Shortle and Meeker (1986). Five multiple mutants in staphylococcal nuclease were constructed from a group of random single mutants that were screened initially for their ability to affect the stability of the enzyme in vivo. The component mutants do not make direct contact with each other in the multiple mutants. Generally, these variants exhibit nearly additive effects except for the double mutant V66L,G88V (Table IV). In addition to those of staphylococcal nuclease, additive effects on the $\Delta\Delta G_{unfolding}$ (assayed by reversible denaturation) have also been determined for the N-terminal domain of λ repressor (one example; Hecht et al., 1986), the α-subunit of *E. coli* Trp synthetase (one example; Hurle et al., 1986), T4 lysozyme (six examples; Wetzel et al., 1988), the gene V product of bacteriophage f1 (one example; Sandberg & Terwilliger, 1989),

Table IV: Comparison of Sums of $\Delta\Delta G_{unfolding}$ from Component Mutants vs the Multiple Mutant

assay	$\Delta\Delta G_{unfolding}$ component mutants			sum	multiple mutant
Staphylococcal Nuclease					
	V66L + G79S[a]				
GuHCl	−0.2	−2.6		−2.8	−3.3
urea	+0.2	−2.9		−2.7	−3.6
	V66L + G88V				
GuHCl	−0.2	−1.0		−1.2	−2.1
urea	+0.2	−0.9		−0.7	−1.4
	I18M + A69T				
GuHCl	−0.6	−2.7		−3.3	−2.8
urea	−0.7	−2.9		−3.6	−3.8
	I18M + A90S				
GuHCl	−0.6	−1.4		−2.0	−2.2
urea	−0.7	−1.4		−2.1	−2.2
	V66L + G79S + G88V				
GuHCl	−0.2	−2.6	−1.0	−3.8	−3.0
urea	+0.2	−2.9	−0.9	−3.6	−3.4
N-Terminal Domain of λ Repressor					
	G46A + G48A[b]				
thermal melt	+0.7	+0.9		+1.6	+1.1
T4 Lysozyme					
	I3C + C54V[c]				
thermal melt	+1.2	−0.7		+0.5	+0.4
	I3C + C54T				
thermal melt	+1.2	+0.3		+1.5	+1.5
	I3C + C54T + R96H				
thermal melt	+1.2	+0.3	−2.8	−1.3	−2.5
	I3C,C54T + R96H				
thermal melt	+1.5	−2.8		−1.3	−2.5
	I3C + C54T + A146T				
thermal melt	+1.2	+0.3	−1.5	0	−0.5
	I3C,C54T + A146T				
thermal melt	+1.5	−1.5		0	−0.5
Bacteriophage f1 Gene V					
	V35I + I47V[d]				
GuHCl	−0.4	−2.4		−2.8	−2.9
Kringle-2 of tPA					
	H64Y + R68G[e]				
thermal melt	+2.9	+0.7		+3.6	+3.4
Turkey Ovomucoid Third Domain					
	G32A + N28S[f]				
thermal melt	+0.8	−0.5		+0.3	+0.2
	Y20H + N45-CHO				
thermal melt	−0.8	+0.3		−0.5	−0.6
α Subunit of *E. coli* Trp Synthetase					
	Y175C + G211E[g]				
GuHCl	−0.1	+0.3		+0.2	−1.3

[a] Shortle and Meeker (1986). [b] Hecht et al. (1986). [c] Wetzel et al. (1988). [d] Sandberg and Terwilliger (1989). [e] R. Kelley, personal communication. [f] Otlewski and Laskowski (1990). N45-CHO refers to a glycosylation of Asn45. [g] Hurle et al. (1986).

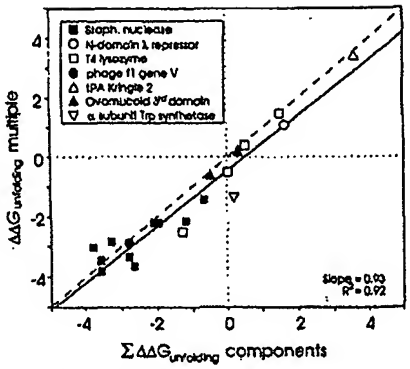

FIGURE 5: Plot showing sum of changes in free energy of unfolding of component mutants and resulting multiple mutant. Data are taken from Table IV and represent staphylococcal nuclease (■), N-terminal domain of λ repressor (○), T4 lysozyme (□), bacteriophage f1 gene V product (●), Kringle-2 domain of tissue plasminogen activator (▲), turkey ovomucoid third domain (△), and the α-subunit of Trp synthetase (▽). The dashed line represents a theoretical line of unity slope, and the solid line represents the best fit.

natural variants of ovomucoid third domain (two examples; Otlewski & Laskowski, 1990), and the Kringle-2 domain of human tissue plasminogen activator (t-PA) (one example; R. Kelley, personal communication).

Collectively, this data set gives a high linear correlation (R^2 = 0.94) and slope near unity (Figure 5). The generally simple additive behavior is somewhat surprising given the highly cooperative nature of protein folding. There are discrepancies in some of the additivity examples besides the staphylococcal nuclease mutant (V66L,G88V). For example, the 1.5 kcal/mol discrepancy for the Y175C,G271E double mutant in Trp synthetase (Table IV) is proposed to result from the fact that these residues are in direct contact (Hurle et al., 1986). Furthermore, proximity effects may account for the large differences between the sum of the component mutants and the multiple mutants for the α-helical double glycine mutant G46A,G48A in λ repressor (Hecht et al., 1986), and when combining R96H with the C3–C97 disulfide mutant in T4 lysozyme (Wetzel et al., 1988). In contrast, an exchange of two side chains that contact one another (V35I and I47V) in the hydrophobic core of the gene V product of f1 phage produced simple additive effects (Sandberg & Terwilliger, 1989; Table IV). It should be noted that this data base exhibiting simple additivity may be biased for single mutants that stably fold, because severely unstable proteins are more difficult to express.

By analogy to transition-state binding effects, one can certainly imagine instances where the stabilizing effects of mutations should reach a plateau. For example, denaturation at high temperatures can become controlled by a chemical step such as deamidation (Ahern et al., 1987), so that additional mutants that stabilize the folded form of the protein may be irrelevant. Another obvious example where complex additivity can be observed in protein stability is the stabilizing effect of disulfide bonds and noncovalent intramolecular contacts that require interactions between two or more residues. In these cases, the stabilizing interaction between two side chains can be broken with only one mutation.

Applications of Additivity in Rational Protein Design

A strategy of additive mutagenesis, where a series of single mutants each making a small improvement in function are combined, is one of the most powerful tools in designing functional properties in proteins. This approach has been remarkably successful in stabilizing proteins to irreversible inactivation, such as λ repressor (Hecht et al., 1986), subtilisin (Bryan et al., 1987; Cunningham & Wells, 1987; Pantoliano et al., 1989), kanamycin nucleotidyltransferase (Liao et al., 1986; Matsumura, 1986), neutral protease (Imanaka et al., 1986), and T4 lysozyme (Wetzel et al., 1988; Matsumara et al., 1989). This strategy has been applied to enhancing the catalytic efficiency of a weakly active variant of subtilisin (Carter et al., 1989), engineering the substrate specificity of subtilisin (Wells et al., 1987a,b; Russell & Fersht, 1987) and the coenzyme specificity of glutathione reductase (Scrutton et al., 1990), designing protease inhibitors with exquisite protease specificity (Laskowski et al., 1989), and recruiting human prolactin to bind to the hGH receptor (Cunningham et al., 1990). In addition, additivity principles have been used to engineer the pH profile of subtilisin (Russell & Fersht, 1987) and to design the affinity and specificity of λ repressor (Nelson & Sauer, 1985).

For this approach to work does not require that all the component mutants act in a simply additive manner but just that their effects accomulate. For example, despite the complex additivity of effects in the catalytic triad of subtilisin, there are mutagenic pathways that are energetically cumulative for installing the triad (Carter & Wells, 1988; Wells et al., 1987c). Starting with the triple mutant S221A,H64A,D32A, there is a progressive enhancement for installing Ser221 (−1.1 kcal/mol), then His64 (−1.0 kcal/mol), and finally Asp32 (−6.5 kcal/mol). Another cumulative pathway of Ser221, then Asp32, and finally His64 is possible if the Ser221,Asp32 intermediate were to use HisP2 substrates (Carter & Wells, 1987). Elaborating such cumulative pathways is important for understanding how a catalytic apparatus may have evolved and is practically useful for considering how to install such catalytic machinery into weakly active catalytic antibodies.

Conclusions

In the majority of cases, combination of mutations that affect substrate or transition-state binding, protein–protein interactions, DNA–protein recognition, or protein stability exhibits simple additivity. Simple additivity is commonly observed for distant mutations at rigid molecular interfaces such as in protein–protein and DNA–protein interactions, where the mutations are unlikely to alter grossly the structure or mode of binding.

Large deviations from simple additivity can occur when the sites of mutations strongly interact with one another (by making direct contact or indirectly through electrostatic interactions or large structural perturbations) and/or when both sites function cooperatively (as for the catalytic triad and oxyanion binding site of subtilisin). Changes at sites that can contact each other do not always lead to complex additivity; this may reflect relatively weak interactions between the two sites or indicate that the interactions are compensatory and appear to be weak.

It is important to point out the magnitude of errors in predicting the free energy effect in the multiple mutant from the component single mutants. Generally, for those cases exhibiting simple additivity (Figures 1, 4, and 5), the discrepancy in free energy between the sums of the components and multiple mutants is about ±25%. Part of this is the result of compounding errors when summing the single mutants, and the rest is presumably due to weak interaction terms. Nonetheless, this means that if the total free energy change is about 3 kcal/mol, the change in the equilibrium constant

(related by $K_{x,y}/K_{wt} = 10^{-\Delta\Delta G/RT} = 155$) will often be off by a factor of 4. Thus, while the free energy effects accumulate, significant deviations will occur in predicting the final equilibrium constants when component mutants contribute a large free energy term.

Simple additivity reflects the modularity of component amino acids in protein function. This results from the fact that the perturbations in energetics and structure resulting from most mutations are highly localized. In the past six years, an additive mutagenesis strategy has been extremely effective in engineering proteins—of course, nature has been using this strategy much longer.

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Registry No. RNase, 9001-99-4; tyrosyl-tRNA synthetase, 9023-45-4; trypsin, 9002-07-7; dihydrofolate reductase, 9002-03-3; subtilisin BPN′, 9014-01-1; glutathione reductase, 9001-48-3; staphylococcal nuclease, 9013-53-0; lysozyme, 9001-63-2; plasminogen activator, 105913-11-9; tryptophan synthetase, 9014-52-2.

REFERENCES

Ackers, G. K., & Smith, F. R. (1985) *Annu. Rev. Biochem. 54*, 597–629.

Ahern, T. J., Casal, J. I., Petsko, G. A., & Klibanov, A. M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 675–679.

Alber, T., Dao-pin, S., Wilson, K., Wozniak, J. A., Cook, S. P., & Matthews, B. W. (1987) *Nature 330*, 41–46.

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5631–5640.

Ardelt, W., & Laskowski, M., Jr. (1990) *J. Mol. Biol.* (submitted for publication).

Bott, R., Ultsch, M., Wells, J., Powers, D., Burdick, D., Struble, M., Burnier, J., Estell, D., Miller, J., Graycar, T., Adams, R., & Power, S. (1987) *ACS Symposium Series 334* (LeBaron, H. M., Mumma, R. O., Honeycutt, R. C., & Deusing, J. H., Eds.) pp 139–147, American Chemical Society, Washington, DC.

Brown, K. A., Brick, P., & Blow, D. M. (1987) *Nature 326*, 416–418.

Bryan, P. N., Rollence, M.-L., Pantoliano, M. W., Wood, J., Finzel, B. C., Gilliland, G. L., Howard, A. J., & Poulos, T. L. (1987) *Proteins: Struct., Funct., Genet. 1*, 326–334.

Carter, P., & Wells, J. A. (1987) *Science 237*, 394.

Carter, P., & Wells, J. A. (1988) *Nature 332*, 564–568.

Carter, P., & Wells, J. A. (1990) *Proteins: Struct., Funct., Genet.* (in press).

Carter, P., Nilsson, B., Burnier, J. P., Burdick, D., & Wells, J. A. (1989) *Proteins: Struct., Funct., Genet. 6*, 240–248.

Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1984) *Cell 38*, 835–840.

Chothia, C., & Lesk, A. (1986) *EMBO J. 5*, 823–826.

Craik, C. S., Largman, C., Fletcher, T., Roczniak, S., Barr, P. J., Fletterick, R., & Rutter, W. J. (1985) *Science 228*, 291–297.

Cunningham, B. C., & Wells, J. A. (1987) *Protein Eng. 1*, 319–325.

Cunningham, B. C., Henner, D. J., & Wells, J. A. (1990) *Science 247*, 1461–1465.

Ebright, R. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 303–307.

Ebright, R. H., Kolb, A., Buc, H., Kunkel, T. A., Krakow, J. S., & Beckwith, J. (1987) *Proc. Natl. Acad. sci. U.S.A. 84*, 6083–6087.

Empie, M. W., & Laskowski, M., Jr. (1982) *Biochemistry 21*, 2274–2284.

Fersht, A. (1985) in *Enzyme Structure and Mechanism*, 2nd ed., Chapters 3, 12, and 13, W. H. Freeman and Co., New York.

Fersht, A. R. (1987) *Biochemistry 26*, 8031–8037.

Fersht, A. R., Wilkinson, A. J., Carter, P., & Winter, G. (1985) *Biochemistry 24*, 5858–5861.

Hecht, M. H., Sturtevant, J. M., & Sauer, R. T. (1986) *Proteins: Struct., Funct., Genet. 1* 43–46.

Horovitz, A., & Rigbi, M. (1985) *J. Theor. Biol. 116*, 149–159.

Howell, E. E., Villafranca, J. E., Warren, M. S., Oatley, S. J., & Kraut, J. (1986) *Science 231*, 1123–1128.

Hurle, M. R., Tweedy, N. B., & Matthews, C. R. (1986) *Biochemistry 25*, 6356–6360.

Imanaka, T., Shibazaki, M., & Takagi, M. (1986) *Nature 324*, 695–697.

Jencks, W. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4046–4050.

Jones, M. D., Lowe, D. M., Borgford, T., & Fersht, A. R. (1986) *Biochemistry 25*, 1887–1891.

Katz, B. A., & Kossiakoff, A. (1986) *J. Biol. Chem. 261*, 15480–15485.

Laskowski, M., Jr., Tashiro, M., Empie, M. W., Park, S. J., Kato, I., Ardelt, W., & Mieczorek, M. (1983) in *Protease Inhibitors: Medical and Biological Aspects* (Katunuma, N., Ed.) pp 55–68, Japan Scientific Societies Press, Tokyo, Japan.

Laskowski, M., Jr., Kato, I., Ardelt, W., Cook, J., Denton, A., Empie, M. W., Kohr, W. J., Park, S. J., Parks, K., Schatzley, B. L., Schoenberger, O. L., Tashiro, M., Vichot, G., Whatley, H. E., Wieczorek, A., & Wieczorek, M. (1987) *Biochemistry 26*, 202–221.

Laskowski, M., Jr., Park, S. J., Tashiro, M., & Wynn, R. (1989) in *Protein Recognition of Immobilized Ligands*, UCLA Symposia on Molecular and Cellular Biology (Hutchens, T. W., Ed.) Vol. 80, pp 149–160, A. R. Liss, New York.

Leatherbarrow, R. J., Fersht, A. R., & Winter, G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7840–7844.

Lehming, N., Sartorius, J., Kisters-Woike, B., von Wilcken-Bergmann, B., & Muller-Hill, B. (1990) *EMBO J. 9*, 615–621.

Liao, H., McKenzie, T., & Hageman, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 83*, 576–580.

Lowe, D. M., Fersht, A. R., Wilkinson, A. J., Carter, P., & Winter, G. (1985) *Biochemistry 24*, 5106–5109.

Matsumura, M., Yasumura, S., & Aiba, S. (1986) *Nature 323*, 356–358.

Matsumura, M., Signor, G., & Matthews, B. W. (1989) *Nature 342*, 291–294.

Matthews, B. W. (1987) *Biochemistry 26*, 6885–6888.

Mayer, R. J., Chen, J.-T., Taira, K., Fierke, C. A., & Benkovic, S. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7718–7720.

Mitchinson, C., & Baldwin, R. L. (1986) *Proteins: Struct., Funct., Genet. 1*, 23–33.

Nelson, H. C. M., & Sauer, R. T. (1985) *Cell 42*, 549-558.
Otlewski, J., & Laskowski, M., Jr. (1990) (submitted for publication).
Pantoliano, M. W., Whitlow, M., Wood, J. F., Dodd, S. W., Hardman, K. D., Rollence, M. L., & Bryan, P. N. (1989) *Biochemistry 28*, 7205-7213.
Russell, A. J., & Fersht, A. R. (1987) *Nature 328*, 496-500.
Sandberg, W. S., & Terwilliger, T. C. (1989) *Science 245*, 54-57.
Sarai, A., & Takeda, Y. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6513-6517.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157-162.
Scrutton, N. S., Berry, A., & Perham, R. N. (1990) *Nature 343*, 38-43.
Shortle, D., & Meeker, A. K. (1986) *Proteins: Struct., Funct., Genet. 1*, 81-89.
Takeda, Y., Sarai, A., & Rivera, V. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 439-443.
Wells, J. A., & Estell, D. A. (1988) *Trends Biochem. Sci. 13*, 291-297.
Wells, J. A., Cunningham, B. C., Graycar, T. P., & Estell, D. A. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5167-5171.
Wells, J. A., Powers, D. B., Bott, R. R., Graycar, T. P., & Estell, D. A. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1219-1223.
Wells, J. A., Cunningham, B. C., Graycar, T. P., Estell, D. A., & Carter, P. (1987c) *Cold Spring Harbor Symp. Quant. Biol. 52*, 647-652.
Wetzel, R., Perry, L. J., Baase, W. A., & Becktel, W. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 875*, 401-405.
Wilde, J. A., Bolton, P. H., Dell'Acqua, M., Hibler, D. W., Pourmotabbed, T., & Gerlt, J. A. (1988) *Biochemistry 27*, 4127-4132.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., & Winter, G. (1983) *Biochemistry 22*, 3581-3586.
Wilkinson, A. J., Fersht, A. R., Blow, D. M., Carter, P., & Winter, G. (1984) *Nature 307*, 187-188.

Accelerated Publications

Role of Tyrosine M210 in the Initial Charge Separation of Reaction Centers of *Rhodobacter sphaeroides*†

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ABSTRACT: Femtosecond spectroscopy was used in combination with site-directed mutagenesis to study the influence of tyrosine M210 (YM210) on the primary electron transfer in the reaction center of *Rhodobacter sphaeroides*. The exchange of YM210 to phenylalanine caused the time constant of primary electron transfer to increase from 3.5 ± 0.4 ps to 16 ± 6 ps while the exchange to leucine increased the time constant even more to 22 ± 8 ps. The results suggest that tyrosine M210 is important for the fast rate of the primary electron transfer.

The primary photochemical event during photosynthesis of bacteriochlorophyll- (Bchl-) containing organisms is a light-induced charge separation within a transmembrane protein complex called the reaction center (RC). The crystal structures of RC's from *Rhodopseudomonas* (*Rps.*) *viridis* and *Rhodobacter* (*Rb.*) *sphaeroides* have been solved to high resolution [reviewed in Deisenhofer and Michel (1989), Chang et al. (1986), Tiede et al. (1988), and Rees et al. (1989)]. The RC from *Rb. sphaeroides* contains three protein subunits referred to as L, M, and H, according to their respective mobilities in SDS-polyacrylamide gels. Associated with the L and M subunits are the cofactors, consisting of four Bchl *a*, two bacteriopheophytin (Bph) *a*, one atom of non-heme ferrous iron, two quinones (Q_A and Q_B), and in some species one carotenoid [reviewed in Parson (1987) and Feher et al. (1989)]. The cofactors are arranged in two branches (Figure 1) with an approximate C_2 axis of symmetry. The kinetic data support a model in which the primary electron transfer proceeds after light absorption by the primary donor [a special pair of Bchl referred to as P; reviewed in Kirmaier and Holten (1987)]. The absorption of light generates the excited electronic state P*, which has a lifetime of approximately 3 ps. An electron is transferred from P along only one branch (the so-called A-branch). It is generally accepted that after approximately 3 ps the electron arrives at the Bph on the A-side (H_A) and after 220 ps it reaches Q_A. The role of the accessory Bchl located between P and H_A (referred to as B_A) has not been definitely assigned. Recently, we have shown that at room temperature an additional kinetic ($\tau = 0.9$ ps) component is detectable (Holzapfel et al., 1989). The spectral properties and the kinetic constants lead to the conclusion that the corresponding intermediate is the radical pair $P^+B_A^-$ (Holzapfel et al., 1990).

Additional intriguing points concerning the process of

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